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Hertta Pulkkinen

The Use of Recombinant Human Type II Collagen for Articular Cartilage Tissue Engineering

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HERTTA PULKKINEN

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ABSTRACT

Adult cartilage has a limited intrinsic capacity to regenerate and heal after injury. Therefore, a range of repair techniques have been adopted in an attempt to restore function and prevent further degeneration and development of osteoarthritis. Tissue engineering (TE) of cartilage using biodegradable material as a scaffold for chondrocytes could help the restoration of functional tissue. In this thesis a novel biomaterial, recombinant human type II collagen (rhCII), was tested as a scaffold for chondrocytes. The rhCII was proven to be a safe material for chondrocytes, and it allowed cell growth and production of cartilage ECM constituents, type II collagen and proteoglycans (PGs). When rhCII was used as a scaffold to repair osteochondral defects in a rabbit model, the repair tissue contained typical articular cartilage ECM and the use of rhCII helped to achieve better filling of the cartilage defect. The rhCII-assisted repair tissue integrated well to the subchondral bone and the tissue showed mechanical durability especially in the deeper zones. However, the repair quality was not much improved when compared to the spontaneous healing process. In addition, the superficial layer of repair tissue showed fibrillation and type I collagen production. Furthermore, the integration into the adjacent cartilage was not complete in either rhCII assisted or the spontaneous repair tissue. Therefore, before rhCII can be used in human patients, further studies concerning enhancing the integration, and preventing surface fibrillation of the repair tissue, are needed.

National Library of Medical Classification: QT 37, QU 55
Medical Subject Headings: Biocombatible Materials; Cartilage, Articular; Collagen type II; Hydrogels; Recombinant Proteins; Tissue Engineering; Tissue Scaffolds

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TIIVISTELMÄ

Nivelruston vaurioituessa sen kyky korjaantua itsestään on lähes olematon. Biohajoavien tukimateriaalien ja solujen käyttö nivelruston kudosteknologiassa voi auttaa rustokudoksen korjauksessa ja toiminnallisuuden palauttamisessa. Tämän väitöskirjan tavoitteena oli testata uutta biomateriaalia: ihmisen rekombinantti tyypin II kollageenia (rhCII) rustosoluille tukimateriaaliksi. Tulokset osoittivat, että rhCII-materiaali oli rustosoluille turvallinen kasvuympäristö, ja sen käyttö solujen tukimateriaalina mahdollisti solujen kasvun ja ravinteiden saannin. RhCII tukimateriaalissa viljellyt rustosolut tuottivat nivelruston soluväliaineelle tyypillisiä aineosia: tyypin II kollageenia ja proteoglykaaneja. Kun rhCII materiaalia käytettiin rustovaurioiden korjaamiseen eläinmallissa, huomattiin että korjauskudoksessa esiintyi rustolle tyypillistä kudosta, korjauskudos kiinnittyi hyvin rustonalaiseen luuhun ja korjauskudoksen mekaaninen kestävyys oli jonkin verran parempi kuin spontaanissa korjauskudoksessa. Lisäksi rhCII paransi rustovaurioiden täyttöastetta. Verrattaessa rhCII:n avulla saatua korjauskudosta spontaaniin korjaustulokseen, ei merkittäviä eroja kuitenkaan havaittu. Lisäksi sekä rhCII ja spontaani korjauskudos olivat pintakerrokseltaan säikeisiä ja sisälsivät rustolle epätyypillistä tyypin I kollageenia. Myös kiinnittyminen viereiseen, terveeseen rustokudokseen oli paikoitellen puutteellinen. Jatkossa tulisi tutkia kiinnittymistä parantavien aineiden käyttämistä sekä mahdollisesti pintakerroksen peittämistä ennen mahdollisia potilassovelluksia.

Yleinen Suomalainen asiasanasto: biolääketiede; biomateriaalit; eläinkokeet; geelit; kollageenit; kudoksensiirto; nivelrusto; solusiirto

To the Unknown

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Kuopio, 2nd of November 2012

Hertta Pulkkinen

List of the original publications

This dissertation is based on the following original publications:

I Pulkkinen H, Tiitu V, Lammentausta E, Laasanen MS, Hämäläinen E-R, Kiviranta I, Lammi MJ: Cellulose sponge as a scaffold for cartilage tissue engineering.
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III Pulkkinen HJ, Tiitu V, Valonen P, Jurvelin JS, Lammi MJ, Kiviranta I: Engineering of cartilage in recombinant human type II collagen gel in nude mouse model *in vivo*. *Osteoarthritis and Cartilage 18(8):1077-1087, 2010.*

IV Pulkkinen HJ, Tiitu V, Valonen P, Jurvelin J, Rieppo L, Töyräs J, Silvast TS, Lammi MJ, Kiviranta I: Repairing osteochondral defects with recombinant human type II collagen gel and autologous chondrocytes in a rabbit model. *Submitted.*

Contents

Abbreviations

3D	Three-dimensional
ACT	Autologous chondrocyte transplantation
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
CCI	Characterized chondrocyte implantation
CD44	Cluster of differentiation 44
cDNA	Complementary DNA
COMP	Cartilage oligomeric matrix protein
CT	Computed tomography
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
e-POL	Enhanced polarized light microscopy
FCS	Fetal calf serum
FT-IRIS	Fourier-transform infrared imaging spectroscopy
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hyaluronic Acid (also called Hyaluronan)
IGF	Insulin-like growth factor
IL	Interleukin

MACI	Matrix-induced chondrocyte implantation
MRI	Magnetic resonance imaging
MMP	Matrix metalloproteinase
MMP13	Matrix metalloproteinase 13 (also called Collagenase-3)
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
OA	Osteoarthritis
OAT	Osteochondral allograft transplantation
PBS	Phosphate buffered saline
PG	Proteoglycan
PLA	Polylactic acid
PEG	Polyethylene glycol
PRG4	Proteoglycan 4
RhCII	Recombinant human type II collagen
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscope
SZP	Superficial zone protein
TE	Tissue engineering
TGF	Transforming growth factor
TIMP	Tissue inhibitor of MMP
TNF	Tumour necrosis factor
VOI	Volume of interest

1 Introduction

Articular cartilage exists as a thin layer of avascular tissue covering the ends of the bones in synovial joints. It is composed of a relatively low number of cartilage resident cells called chondrocytes. These cells produce the extracellular matrix (ECM) that is specific to this tissue, which mainly contains type II collagen and proteoglycans (PGs) (Buckwalter 1998b). Normally functioning cartilage is required for the flexible movement of joints. However, if adult cartilage is damaged, it has a limited intrinsic capacity to regenerate and heal (Newman 1998). Cartilage injuries cause pain and immobility and can lower the ability of the joint to function and furthermore debilitate the quality of life. If left untreated, cartilage defects can predispose the injured person to the development of a much more severe condition, osteoarthritis (OA) (Brown 2006, Messner 1996).

However, since spontaneous repair of articular cartilage is poor, several methods have been developed in order to improve it's healing. Conventional methods to repair cartilage defects include different surgical procedures; such as drilling and shaving, as well as microfracturing (Hunziker 2002). These methods are based on alleviating pain with improved joint mobility, and on findings that bone marrow stimulation can promote growth of new tissue. However, the outcome in these techniques differs amongst patients, and many unsatisfactory results have been reported.

Due to the development of cell culturing and biomedical materials engineering, a new approach to regenerative medicine has emerged. Tissue engineering (TE) of cartilage seeks to aid the regeneration or repair of articular cartilage in the defective areas by using transplanted cells and biocompatible materials in an effort to grow new functional tissue. At this moment TE is considered a promising solution to repair cartilage defects and to produce sustainable regenerative tissue. A wide variety of materials have been tested as scaffolds for chondrocytes, but perhaps the most promising ones are natural materials that could offer a surrounding that mimic the innate environment of the cells. One such molecule is type II collagen.

Type II collagen has been tested as a scaffold for chondrocytes with promising results in rabbits and dogs for articular cartilage TE as previously reported (Funayama 2008, Lee 2003, Nehrer 1998). The collagens that are currently used are of animal origin, and therefore raise questions about safety (Lynn 2004). As an alternative source for collagen,

a technique to manufacture human recombinant collagen in yeast cultivations (*Pichia pastoris*), has been developed (Myllyharju 2000). This manufacture process has been shown to produce hydroxylated triple helical collagen that builds up a structure with a thermal stability similar to native collagen when coexpressed with recombinant prolyl hydroxylase (Baez 2005).

The aim of this thesis was to investigate the properties of recombinant human type II collagen (rhCII) as a novel biomaterial to be used as a scaffold for chondrocytes in articular cartilage TE. This rhCII material was first tested as a coating material for cellulose scaffolds, and was demonstrated to improve the chondrocyte attachment in these scaffolds. The feasibility of the rhCII material was then investigated as a gel-like formula, first in cell culture conditions *in vitro*, and then in animal models *in vivo*. Various methods to analyze the cellular properties and matrix production of chondrocytes cultivated in rhCII, and furthermore the tissue structure and mechanical properties of the TE rhCII constructs, were applied. The results obtained in this thesis provide important information about cartilage repair and the limitations of the current techniques.

2 Review of the literature

2.1 ARTICULAR CARTILAGE

Articular cartilage, also called *hyaline cartilage*, is an avascular form of connective tissue originating from mesenchyme. It is located in synovial joints, forming a thin cartilaginous layer covering the articulating surfaces of the bones (Figure 1). The synovial joints are closed systems, encapsulated by a synovial membrane. The cells in the synovial membrane excrete synovial fluid, which together with the articular cartilage allows nearly frictionless movement of the enclosed joints.

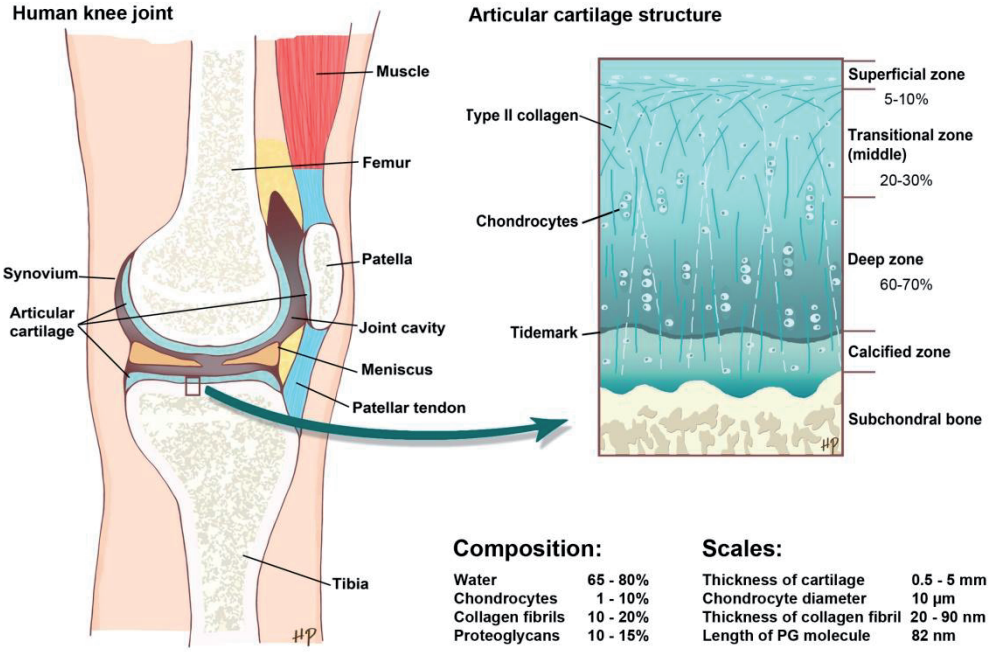

Figure 1. A schematic representation of the structure of a human knee joint, and a larger magnification of the structure of the articular cartilage. The articular cartilage (light blue in figure) is located on the surfaces of the articulating bones. The joint capsule encloses the joint cavity. The structure of articular cartilage consists of chondrocytes and an extracellular matrix: type II collagen and proteoglycans organized in a depth-dependent manner (Newman 1998). The collagen fibrils are arranged as an arched structure, and the arch-like organization is shown in the picture as dotted white lines.

The role of articular cartilage as a load-bearing tissue has a major impact on the structure and function of this tissue. Articular cartilage is composed of a relatively small fraction of cells, called chondrocytes, within a large amount of extracellular matrix (ECM, Figure 1). The main components of the solid ECM are type II collagen and proteoglycans (PGs). However, the greatest constituent of the extracellular tissue is water.

2.1.1 Chondrocytes

In addition to the recently discovered progenitor cells (Dowthwaite 2004), the predominant cell type found in normal articular cartilage is the chondrocyte. They are rather low in their numbers, and occupy only 1-10% of the tissue volume (Stockwell 1967). Although adult chondrocytes do not divide after maturation in normal articular cartilage (Aigner 2001), they interact actively with the ECM throughout their life. The interaction is mediated via different ECM-binding, cell surface receptors, such as integrins (Salter 1992) and CD44 (Aguiar 1999) that connect the cells to ECM (Figure 2). Interactions and adhesions to the ECM are crucial for cell survival. Integrin-mediated signals block apoptosis and stimulate cell cycle progression amongst other things (Hirsch 1997, Knudson 2002). Body movements change the loading in the cartilage, and this further generates deformation and adjustment in the ECM molecules. These mechanical movements of the ECM molecules activate the ECM-binding receptors (Fitzgerald 2004). The activation of the ECM-binding receptors generates intracellular signaling pathways, which lead to alterations in the gene expression and eventually production of desired proteins (Hynes 2002) or changes in the cell shape (Knudson 2002). The mechanism of mechanotransduction is still not fully understood (Knudson 2002). Changes in other mechanical, electrical or physiochemical factors also affect the metabolism and function of chondrocytes (Urban 1994).

In addition to mechanotransduction, several biologically active molecules have a profound influence on the function of the chondrocytes. These include growth hormones, such as transforming growth factors (TGF-β), bone morphogenetic proteins (BMPs) (Hanada 2001) and insulin-like growth factor-1 (IGF-1) (Smith 2000), as well as cytokines including interleukins (IL-1) and tumour necrosis factor (TNF-α) (Saklatvala 1986). Combined with the information from the ECM receptors, these factors either induce or suppress the production of ECM molecules or ECM degrading enzymes. This constant monitoring and adaptation leads to modification of the ECM when needed. However, in

normal mature cartilage, remodeling is a constant but minimal process, and it is during the growth or disorders such as osteoarthritis, when the production and degradation of the ECM are most active (Goldring 2006).

Due to the lack of blood vessels in cartilage, nutrients and oxygen, originating from the synovium and mediated via the synovial and extracellular fluids, reach the cells by diffusion (O'Hara 1990). This leads to the fact that the oxygen levels of cartilage are rather low, especially in the deep regions (Treuhaft 1971). This means that these cells derive most of their energy by anaerobic glycolysis (Gibson 2008). The rate of chondrocyte activity and metabolism is also related to age (Martin 2002). Once an individual reaches skeletal maturity, the cellular processes and matrix production slow down. For a long time, it was not clear whether the cells in mature animals can divide or not (Mankin 1982). However, since the development of cell culturing techniques, it has been confirmed that adult animal derived chondrocytes can also divide, if the cells are isolated and cultivated in cell culture conditions.

It was long presumed that the chondrocytes are the only cell type that populates the cartilage tissue, but recent investigations show, that a group of stem/progenitor cells are found in the cartilage tissue, especially in the superficial layer (Dowthwaite 2004, Hattori 2007). The exact function and purpose of these cells still remains under speculation, but the surface cells are reported to actively express many growth factors, exhibit plasticity in differentiation, and they may have an important role in the appositional growth of the articular cartilage (Dowthwaite 2004).

2.1.2 Cartilage collagens

Collagens are essential for the structural integrity and stiffness of ECM, and they are the major molecular component of the articular cartilage, amounting 50-80% of the dry weight (Mow 1992). The most abundant collagen in the articular cartilage is fibrillar type II collagen (90-95% of collagens). However other fibrillar or non-fibrillar collagens can be found, (e.g., types III, VI, IX, X and XI) in smaller amounts (Eyre 2002).

Type II collagen possesses a rod-like structure that is composed of three identical intertwined polypeptide chains, which are called collagen α1(II)-chains. These α-chains are comprised of amino acids in repeating sequences of glysine-X-Y, where X is very frequently proline and Y is 4-hydoxyproline. The biosynthesis of collagen requires many modification phases, both inside and outside of the cell (Figure 3). In the beginning of the process the specific gene, in the case of type II collagen, *COL2A1*, is transcribed and processed into messenger RNA (mRNA). Then the translation of the mRNA and production of the α-polypeptide chains takes place in membrane-bound ribosomes. Post translational

modification of the freshly synthetized chains starts right after translation. (Prockop 1995).

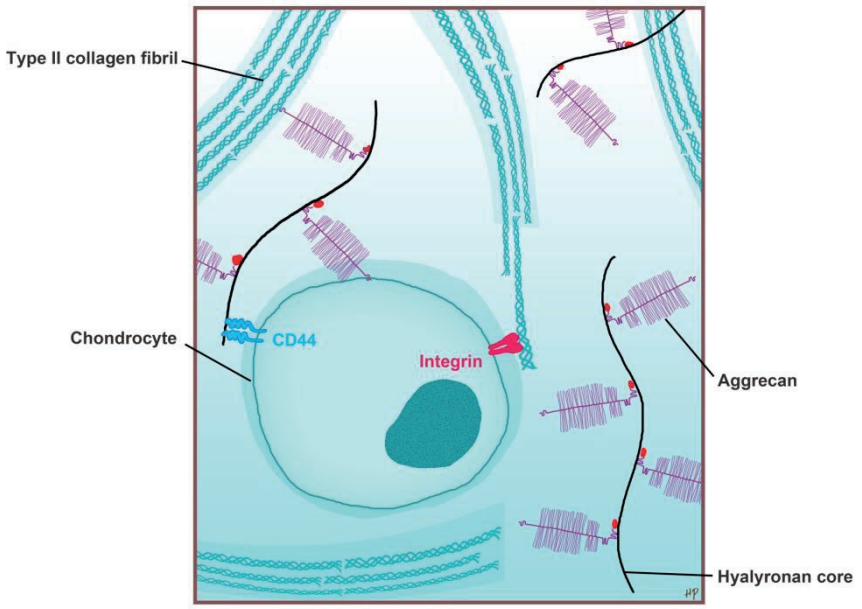

Figure 2. Chondrocyte and ECM molecules. The cell surface receptors, such as CD44 and integrins, are essential for cell survival and maintenance. CD44 binds to hyaluronan, and integrin receptors to collagens. Several aggrecan PGs, often more than 50, are connected to a hyaluronan core, forming large PG aggregates. Type II collagens exist in large collagen fibrils. Also several other PGs, and non-collagenous proteins, exist in the ECM but are not shown in the picture.

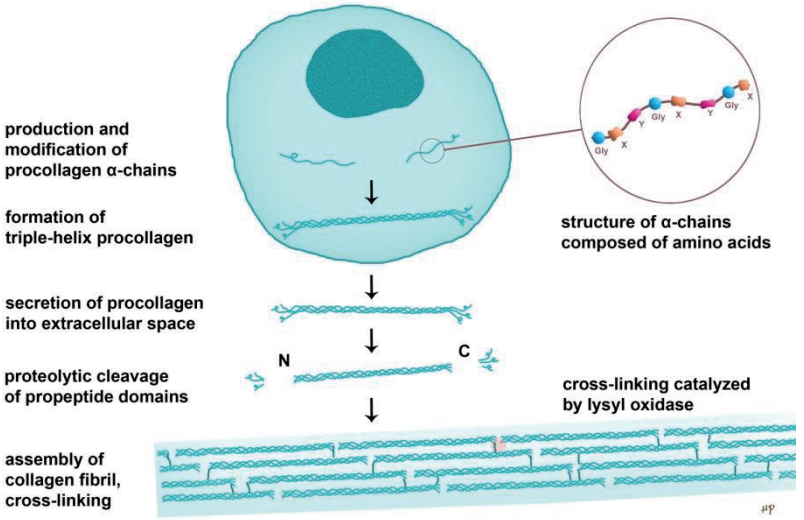

Figure 3. The collagen production by chondrocytes and self-assembly of

collagen fibrils. First the synthetized α-chains form the triple-helix procollagen, which is then secreted and modified outside the cell. After proteolytic cleavage of propeptide domains the collagen (tropocollagen) molecules assembly and form a collagen fibril, a structure that is stabilized with cross-linking, hydrogen bonds and water bridges. Figure is inspired by the review by Kadler et al (Kadler 1996).

The modification of the procollagen α-chains in endoplasmic reticulum allows them to form a folded triple helix structure in which three α-chains are united. At this point, the structure undergoes further post-translational modifications before it is secreted outside the cell via secretory vesicles. The secreted procollagen molecules contain flanking non-collagenous propeptide domains, which are proteolytically cleaved by specific proteinases during the extracellular maturation process, leaving short N- and C-terminal telopeptides on both ends of the molecule. (Prockop 1995). The remaining telopeptide structures serve as a linking site for neighbouring collagen molecules to connect with each other (Miyahara 1982). When the triple-helical collagen molecules are connected with each other as a bundle, a large collagen fibril is formed. This process takes place spontaneously at physiological pH due to the hydrophobic properties and negative charges in certain areas of the chains. The formation of hydrogen bonds and water bridges between the chains further strengthens this structure (Brodsky 2005), together with cross-links that are formed between lysine redidues that are catalyzed by lysyl oxidases (Prockop 1995). Type II collagens are also cross-linked with other collagens, mainly type IX and XI, which help to stabilize the network structure (Eyre 2002).

The structure of collagen fibrils varies in the different areas of the cartilage ECM. Near the cells in the pericellular region the fibrils are thinner, around 20 nm in diameter. Elsewhere, in the ECM, the fibrils are thicker, approximately 60-90 nm in diameter (Holmes 2006, Minns 1977). As previously mentioned, type II collagen molecules interact with another fibrillar collagen (type XI) and with a fibril-associated collagen (type IX) in normal cartilage. The amount of type IX and XI collagens in proportion to type II collagen and is at it's highest in the thinnest, pericellular fibrils (Eyre 2002). These collagen interactions are believed to play a role in regulation of cartilage collagen fibril diameter and in stabilization of the fibrillar network (Eyre 2002, Kadler 1996). Besides that, the PGs in the ECM interact with the type II collagen and play a role in the interfibrillar connections (Scott 1988, Vynios 2001). Collagen (hetero)fibrils are arranged in the ECM as a network (Figure 1), and the orientation of the fibrils changes in relation to the depth and location of

the tissue (Buckwalter 1998b). The network is arranged when several collagen fibrils assembly in paraller direction and form large bundles. The process of fibril assembly and organization is still poorly understood at the moment.

Type II collagen fibrils are stable structures and are resistant to most proteinases. The type II collagen produced during growth can exist for a very long time, lasting even decades. Thus, in normal adult cartilage, the synthesis of new type II collagen is minimal (Aigner 1992, Gebhard 2003). Collagen degradation can be achieved by specific proteolytic enzymes, called collagenases, which are produced by chondrocytes. Collagenases cleave the collagen molecules into shorter fragments. The specific collagenase for type II collagen is collagenase-3 (also called MMP13), a member of the matrix metalloproteinase (MMP) family. Additionally, MMP-1 and MMP-8 have been shown to degrade type II collagen. (Dahlberg 2000). In normal conditions, the occurrence of MMP13 in cartilage is low, but during dynamic processes such as growth (Inada 2004) and disease, for example, osteoarthritis (Dahlberg 2000, Reboul 1996), the production of MMP13 is elevated, and tissue degradation is accelerated. The degradation of collagens is further balanced by secretion of tissue inhibitors of MMPs (TIMPs) that are upregulated by growth factors and block MMP activity (Cawston 1999).

2.1.3 Proteoglycans and other matrix molecules

Inside the collagenous network, there is a vast amount of other non-collagenous proteins, mostly PGs. These have an important role in the function of articular cartilage, especially in providing the osmotic resistance to the tissue under the loading stress. Studies show that PGs compose of around 30% of the dry weight of the articular cartilage (Buckwalter 1998b). They are molecules that contain a core protein, onto which one or more carbohydrate side chains of glycosaminoglycans (GAGs) are attached. The GAGs are defined as long non-branched polysaccharide chains that contain successive disaccharide units consisting of an amino sugar (N-acetyl glucosamine or N-acetyl galactosamine) in one position, and uronic acid or galactose in the other. In cartilage, the GAGs are mainly chondroitin sulphate, keratan sulphate, dermatan sulphate, and hyaluronan (HA). HA is the only GAG, which is not bound to a core protein (Lash 1983).

The major PG in articular cartilage is a large aggregating molecule called aggrecan. Other aggregating molecules are versican and link protein. There are several other PGs in the cartilage ECM, some of the most studied include decorin, biglycan, lumican and fibromodulin. Each PG is identified by the amount and quality of GAGs attached to the core

protein, for example, aggrecan has a large amount of chondroitin sulphate and keratan sulphate side chains. Aggrecan exists in the ECM as PG aggregates; each aggregate is composed of a HA central filament with up to 100 aggrecan molecules attached to it (Figure 2). This structure is further stabilized by a link protein, a protein that has the capacity to bind both aggrecan and HA. (Roughley 2006).

PG biosynthesis involves the synthesis of the core protein and post-translational addition of GAG chains in the Golgi complex (Hascall 1988). The newly completed PGs are then secreted into extracellular space where possible extracellular maturation, interaction with the ECM and aggregation with HA takes place. The synthesis and catabolism of aggrecan by chondrocytes is responsive to several soluble mediators, as well as to mechanical loading (Vertel 2000). Unlike collagens, which are fairly stable in their structure, aggrecans and some other PGs are constantly synthesized and degraded in the tissue. The enzymatic degradation of aggrecan is processed mainly through matrix metalloproteinases (MMPs) and aggrecanases (Sztrolovics 1997).

There are also other proteins that are neither collagens nor PGs, in the cartilage ECM. Perhaps the best known is the cartilage oligomeric protein (COMP).

In their normal state, the GAGs are negatively charged and, therefore, they attract cations near them. The increased osmolarity (concentration of solutes) of the tissue draws water inside the cartilage ECM and creates swelling pressure in the tissue. In turn, the osmotic swelling of the tissue is prohibited by the collagen fibrils. These two counter-balancing forces, the osmotic swelling and the elastic stretching of the collagen fibrils, determine, and have major impact on, the function and properties of articular cartilage as a tissue (Mow 1999).

2.1.4 Structure and organization

Articular cartilage exists as a relatively thin tissue layer. The thickness of this tissue varies regionally and depends on the magnitudes of loads in different regions and joints. For instance, in a heavy weight-bearing joint, such as the knee, the thickness can vary between 1-3 mm in the human femur and tibia and up to 5 mm in the patella (Shepherd 1999). The chondrocytes and ECM molecules are distributed throughout the cartilage in a depth- and site-dependent manner (Figure 1). Everywhere in between the solid matrix molecules there is a large amount of water that can constitute as much as 80% of the wet weight of articular cartilage. Based on histological analysis, the articular cartilage has been divided into four different zones: superficial, transitional (middle), deep (radial), and calcified cartilage (Figure 1). The tissue is not homogenous; chondrocytes and ECM molecules differ in their morphology and density in different zones. (Newman 1998).

The superficial zone is further divided into two layers: a topmost acellular and nonfibrous, *i.e.* amorphous layer (Buckwalter 1998b, Jurvelin 1996), and a deeper layer of flattened chondrocytes (or progenitor cells) with a dense matrix of collagen fibrils arranged parallel to the surface (Buckwalter 1998b). The supercifial zone presumably has great importance to the compressive behavior and function of articular cartilage. It has a major influence on the lubrication mechanism operating between the joint surfaces, and acts as an outer barrier for the cartilage tissue. The collagen fibrils there are organized in parallel fashion to offer a greater strength and tensile stiffness to this upper zone of cartilage. In this zone, the chondrocytes/progenitor cells synthesize less ECM molecules than in other zones, and the water content is high. (Buckwalter 1998b).

In the transitional (middle) zone the chondrocytes are rounded or spherical in their shape, and they are more active and synthesize more ECM with thicker collagen fibrils and a higher PG content. The deep zone is characterised by spheroidal shaped chondrocytes aligned into columns perpendicular (radial) to the joint surface. This zone has the highest amounts of PGs and the lowest concentration of water. Also in this layer, the collagen fibrils are organized in a radial network. In the bottom of the deep zone lays a histologically detectable basophilic thin layer called the “tidemark”. Under the tidemark is a relatively thin zone of calcified cartilage. The cells and matrix there form the boundary between the upper uncalcified cartilage and the subchondral bone. (Buckwalter 1999).

In addition to the four horizontal matrix subdivisions mentioned above, cartilage tissue ECM can also be identified in its relation to the chondrocytes, these regions are: the pericellular region (closest to the

cells), the territorial region (surrounding pericellular region), and the interterritorial region (rest of the tissue). Around the cells, in the pericellular region, there are no thick collagen fibrils present, but a large amount of PGs, hyaluronan and thin fibrils of type VI collagen. The pericellular area is surrounded by pericellular capsule, forming a structural and functional unit called the *chondron*. The chondron area is the active environment of the cell, where the interaction with the matrix happens. (Poole 1997). In the pericellular layer the composition and properties of matrix differ from the surrounding ECM, and therefore it has different elastic properties and a significant role in altering the principal stress and strain magnitudes within the chondrocytes (Guilak 2000).

2.1.5 Function and biomechanical properties

The location of articular cartilage between the bones in diarthrodial (synovial) joints highly determines the purpose of the tissue, that is, to withstand and transmit mechanical forces emerging from motions. In order to fulfill this task, the articular cartilage must perform functions related to lubrication and wear-resistance. The lubrication and minimization of friction is mediated by two main mechanisms. The cells of the synovium secrete synovial fluid into the joint cavity, and this fluid together with pressurized tissue fluid results in the formation of fluid film between the joint surfaces during high loads. A small glycoprotein called *lubricin*, (also known as superficial zone protein, SZP, or proteoglycan 4, PRG4), is secreted by synovial cells and chondrocytes, and plays an important role in the lubrication. It is a component of the synovial fluid and also exists attached to cartilage surfaces. There it acts as a lubricant, protects the surface chondrocytes and prevents an abnormal growth of the cells (Rhee 2005). At low loads, the load is supported by surface-to-surface contact, and the lubrication is due to the lubricant surface molecules (Schmidt 2007). All of the tissue components, the chondrocytes and the ECM, including the water, collagens and PGs, have a special, interconnected role in the weight-bearing capacity of the tissue.

The collagen fibrils arranged in an arch-like network form the structural backbone and scaffold of the articular cartilage (Chen 1998). The collagen fibrils have good tensile strength. They enable resistance to the deformation of the tissue, which is created by the external mechanical load and internal osmotic pressure. The PGs that are mechanically or chemically bound to collagen and immobilized inside the cartilage ECM contribute to the formation of the osmotic pressure by attracting water molecules inside the tissue. But unlike the stabilized, large PGs, the water

can flow freely through the tissue and this has a major effect on the mechanical function of articular cartilage.

Charasteristics of the water flow and the mechanical properties of cartilage differ depending on the rate of the loading. Under rapid loading, there is no time for the water to move, therefore, the arrangement of collagen fibrils withstands the load and cartilage behaves as an incompressible elastic solid (Suh 1995). However, during a constant and continued load, the water starts to flow and relocate inside the tissue, and out of the cartilage, resulting in compression. Initially, the water flow is relatively rapid under loading, but as a function of time, it slows down and eventually *mechanical equilibrium* is achieved when the deformation ends. Since the GAGs are responsible for attracting water into cartilage, the GAG content and properties affect the capacity to restrict tissue fluid flow and to resist tissue deformation, especially in static compression. If the loading of the cartilage tissue is too high, exceeding the bearing capacity of the tissue, it can cause tissue damage, such as collagen fibril breakdown, loss of PGs and chondrocyte apoptosis or necrosis. Furthermore, the damaged tissue becomes even more vulnerable to mechanical loading, because of the increased permeability and decreased ability to adjust to compression. In that regard, the measurement of cartilage mechanical properties can be used to evaluate the quality of the tissue.

The mechanical properties of cartilage can be studied by applying loading to cartilage and measuring its deformation. Usually cartilage is studied using unconfined compression, confined compression or indentation measurements. For all of these loading geometries three different loading schemes can be used for measurements: stress-relaxation, creep test or dynamic test. For the computation of the results, the structure of the cartilage has to be simplified and the results of the experiments need to be analysed using numerical or analytical models. One of the classical models is the *biphasic model,* where the solid components of cartilage are put together to constitute the solid phase of the tissue, and the interstitial fluid forms the fluid phase (Myers 1983). Since then, more sophisticated models have been developed, which also take into account the organization of the ECM and fibril properties (Korhonen 2003).

2.2 CARTILAGE DEVELOPMENT AND GROWTH

The maintenance, turnover and development of articular cartilage are highly regulated and are influenced by mechanical forces. These forces, such as pressure and shear, influence the expression of gene's that regulate differentiation, growth and matrix production of the chondrocytes. The development of cartilage can be regarded as having two parts; a prenatal phase of differentiation, expansion and rapid growth, and a postnatal period of tissue maturation, during which major modifications take place. The mechanisms and circumstances during normal development have been widely investigated, in order to gain a deeper sight into cartilage repair or regeneration. Information about normal development should also be taken under consideration when cartilage tissue engineering methods are designed.

2.2.1 Prenatal development

During embryogenesis, the cells from the early mesenchyme undergo several changes that lead to their differentiation into chondrocytes, a process called *chondrogenesis*. Once the chondrocytes are differentiated, they start to produce their characteristic ECM: type II collagen and PGs. (Goldring 2006). This complex process of growth and progression is precisely arranged through several growth factors and other signaling molecules, such as BMPs, that guide the cells (Barna 2007). Early chondrocytes start to produce the outline of the skeletal system by producing more ECM and dividing rapidly. At this point the chondrocytes are building up a precursor tissue, which serves as a transient model for the long bones of the body. This hyalinous model begins to rapidly grow and elongate through cell divisions and active ECM production. (Ferguson 1998). Several mediators work together and cause the central cells to undergo further differentiation. Many of these growth factors and transcription factors are mechanosensitive, which means their expression is influenced by the local mechanical environment (Carter 2004). In addition, oxygen levels affect the fate of the differentiating cells. Eventually, the centrally located chondrocytes begin to undergo hypertrophy and produce type X collagen. (St-Jacques 1999). The chondrocytes on the periphery continue their rapid growth, but at the same time the hypertrophic cartilage is invaded by blood vessels and bone forming cells, *osteoblasts*, that start to create the mineralized bone tissue. This process of bone formation, called *endochondral ossification*, starts in the central regions. As the process of bone formation progresses, the cartilaginous precursor model is then almost totally replaced by bone. The only cartilaginous remnants are the growth plate, also known as the

physis, which is left inside the bony structure in proximity to each of the bone ends, and the articular cartilage, that now covers the ends of the bones (Goldring 2006).

Compared to the absence of growth in the adult cartilage, this rapid progress of development is striking, and it has been broadly investigated (for reviews, see (DeLise 2000, Goldring 2006)). During fetal development, the chondrocytes produce ECM that is nonhomogenous and has randomly distributed collagen fibrils (Hunziker 2007). Initially, the collagen fibrils are also few in number, as well as thin in diameter (Keene 1995). In the fetal articular cartilage, the water content is higher, cellularity much denser, collagen content lower, and the tissue much softer than in the adult articular cartilage (Williamson 2001).

2.2.2 Post natal development and maturation

After birth, the transient cartilage that is still present in the growth plates continues to grow and, at the same time, is gradually replaced by the bone. This process enables the elongation of the long bones after birth, and it continues until skeletal maturity is reached. Also the articular cartilage covering the ends of the bones undergoes several changes, during which the originally soft and homogenous tissue matures into durable cartilage with zonal organization. This postnatal development has been suggested to take place in close relation to the bone formation and in fact, this cartilage layer may act as a surface growth plate for the underlying subchondral bone (Hunziker 2007). Nevertheless, the development of bone and cartilage occurs in close proximity and in relation to each other. When skeletal maturity is reached, the individual's longitudinal growth ceases, which happens at the same time as the maturation of articular cartilage. It is noteworthy that cartilage in different joints of the body appears to be rather similar during fetal development, and it is after the birth that regional and joint-dependent differences arise, in response to the differences in loading stress (Williamson 2001).

During growth, both the thickness and water content of the articular cartilage decrease (Julkunen 2009, Rieppo 2009, Williamson 2003), while the collagen content rapidly increases (Julkunen 2009, Rieppo 2009). These changes are a result from gradual adaptation to the joint movements and weight-bearing. At least in some species, the chondrocytes have been detected to proliferate after birth, especially in the superficial layers (Archer 1994). However, in general, proliferation of the chondrocytes ceases after birth and the amount of cells in relation to the ECM is decreased due to the massive production of the latter

(Williamson 2001). Gradually, the chondrocytes also begin to form groups and columnar structures, as the thickening ECM encloses the chondrocytes (Clark 1997).

The collagen fibrils and network have been shown to undergo significant changes during maturation. Step by step, the collagen network develops from the homogenous and parallel-to-surface orientated structure into the mature arch-like orientation (Julkunen 2010, Rieppo 2009). This transformation of the finely organized network of collagen fibrils contributes to the formation of the zones of the cartilage, and it is essential for the proper function of articular cartilage (Julkunen 2010). Previously it was assumed that this reorganization is achieved by internal remodeling, but some speculation exist whether the maturation of collagen fibrils is instead a progression of tissue resorption and neoformation in a process where completely new tissue replaces the original cartilage during maturation (Hunziker 2007).

It appears that the amount of PGs per volume does not vary in a large scale during different developmental stages from newborn to adult (Williamson 2001) or the amount of PGs slightly decreases (Roughley 1980, Williamson 2003). Structural variations have also been detected, including decrease in the size of the PG subunit, change in the relations of GAGs and modifications in the GAGs during maturation (Roughley 1980).

All these changes form a response to the loading of the joints, and contribute to the mechanical properties of the articular cartilage. A significant increase in the tensile stiffness during the maturation has been measured, and it follows the increased collagen content and modifications (Williamson 2001, Williamson 2003). Also the compressive properties undergo significant alterations. The articular cartilage has been shown to become stiffer during maturation (Julkunen 2009, Williamson 2001), as the permeability of the tissue decreases simultaneously (Williamson 2001). These changes in the compressive properties correlate with the GAG content (Sah 1997), or collagen content (Williamson 2001), and it is probable that both of these constituents contribute to the stiffening of the material.

Several studies show that the amount of exercise has significant effects on the quality of articular cartilage, especially in the youth (Helminen 2000). Moderate exercise is considered to be beneficial for developing cartilage (Kiviranta 1988), while too strenous (Arokoski 1994) or too little (caused by immobilization) (Haapala 1999, Jortikka 1997) of physical exercise can weaken the quality of this tissue.

2.3 CARTILAGE INJURIES AND REPAIR

If articular cartilage is damaged, it does not regenerate spontaneously in adults. Sometimes repair tissue may be produced, but the quality of the repair tissue is often fibrous, and it begins to degrade later on. Whereas cartilage injuries are rather common, a range of repair techniques have been adopted in attempt to restore function, relieve patient's pain and prevent further degeneration and development of osteoarthritis.

2.3.1 Cartilage injuries and spontaneous repair

Technically, cartilage is injured if the capacity of the articular cartilage to withstand mechanical forces is exceeded. The capability to bear mechanical loads differs between individuals, and is subjected to temporal and long-term changes during growth, maturation and aging. Several factors can weaken the quality of the cartilage, such as developmental disorders (Buckwalter 1998a), excessive exercise or immobilization (Arokoski 1994, Haapala 1999), and these can predispose cartilage to injuries. However, also healthy cartilage may be injured during high-intensity impact that directly, or indirectly in conjunction with other impairments of joints, such as meniscal or ligamental ruptures damage the cartilage (Buckwalter 1998, Johnson 1998). The incidence of cartilage defects is rather high. According to a large study, which evaluated knee arthroscopies, 63% of the patients had lesions in hyaline cartilage (Curl 1997). Cartilage injuries can vary from minor damage of the cells or tissue, without disruption of the cartilage surface, to more severe fractures and lesions that tear the cartilage (Buckwalter 1997). The clinical symptoms and repair process in these injuries differ in relation to the magnitude and depth of the injuries (Newman 1998). Cartilage lesions are referred as *chondral,* if the cartilage tissue is injured, but the subchondral bone plate is intact. Chondral injuries can be of partial or deep thickness. If the injury penetrates through the whole cartilage layer down to the underlying subchondral bone, the defect is called an *osteochondral lesion*. Whilst smaller, non-distruptive traumas can be asymptomatic, usually the more severe chondral and osteochondral defects cause clinical symptoms, such as swelling, locking of the joint, activity-related pain and dysfunction in joint movement to varying extents.

There are certain processes that take place after chondral injury. Chondrocytes close to the margins of the trauma area have shown to undergo necrosis and or apoptosis (D'Lima 2001a, Kurz 2004). These findings are associated with increased levels of matrix-degrading

enzymes, which have been measured from the synovial fluid (Tchetverikov 2005), and a decrease in the production of ECM molecules, such as type II collagen and aggrecan (Kurz 2005, Lee 2005). Increased production of catabolic enzymes leads to elevated amounts of loose GAGs (D'Lima 2001a) and type II collagen degradation products are released into the synovial fluid (Lohmander 2003). Althought there are several degrading processes going on after cartilage injury, certain constructive actions also take place. It has been shown that individual chondrocytes near the chondral defects are activated, start to proliferate and produce new ECM to some extent. In addition some repair cells are reported to show up and cover the surface of the lesion (Hunziker 1998, Newman 1998). Unfortunately, these events appear to be temporary and do not lead to significant healing. These migrating repair cells were originally thought to be from synovium (Hunziker 1998), but recent findings imply that they can also be identified as the chondrogenic progenitor cells that originate from the superficial layers of cartilage, which activate during injury (Hattori 2007, Seol 2011).

The damage of the integrity of the collagenous network and loss of PGs, either directly by the mechanical injury or during post-traumatic catabolic processes, highly affects the tissue. As a consequence the mechanical properties of the cartilage change causing failure of function (Lu 2008, Quinn 1998). There is evidence that small chondral defects do not fill up with repair tissue in adults (Buckwalter 1997, Kim 1991). There is some controversy about how the injury then develops; it is either thought to lead to further destruction of the cartilage (Brown 2006), or the injured tissue has been reported to remain stable over time (Newman 1998). In conclusion, the chondral injuries induce several catabolic and anabolic reactions, but these changes in metabolism are not helpful in the regeneration of the tissue, and the spontaneous repair remains poor or non-existent.

Osteochondral defects penetrate the subchondral bone and therefore, gain access to the bone marrow cavity and the multipontent mesenchymal stem cells (MSCs) in there. In this case, the small arteries in the bone are violated and this allows blood to flow in, and the formation of a fibrin clot further traps the cells into the defective site (Newman 1998). After a while, a spontaneous repair tissue resembling hyaline cartilage appears (Shapiro 1993). However, the repair seems to be only transient. As time goes on the repaired tissue gradually becomes fibrous, the chondrocytes alter their production of type II collagen into type I, and eventually the new tissue degenerates over time. (Shapiro 1993). Furthermore, the integration of the repair tissue into the adjacent cartilage is usually partial. Finally, many other factors restrain the

regeneration and re-construction of new tissue, such as the anti-adhesive properties of the PGs (Englert 2005, Schaefer 2004).

Major factors that limit the repair in the chondral lesions are 1) the localization of chondrocytes trapped in the ECM and their inability to migrate into the defect, 2) failure of other migrating cells to adhere and produce new tissue, and 3) lack of blood vessels that bring along cells, cytokines and growth factors. But as previously shown, not even the occurrence of blood and proliferative cells does not usually have the desired effect (on the osteochondral defects), and there must be also other factors that diminish the capability of articular cartilage to repair. One of them might be the secretion of the catabolic, degrading enzymes (Tchetverikov 2005) that are on the one hand needed for the modification of the injured tissue, but at the same time can be harmful for the generation of new tissue. The difficulties in the integration of the repair tissue into the adjacent native tissue are easier to understand, when considering the arch-like structures of the collagen fibrils and entrapped PGs. Succesful integration would require novel collagen molecules to protrude into the existing cartilage in a very delicate and site-dependent manner that would allow the anchoring of the new tissue into the native one and permit fusion between the two.

It is possible that there are several factors that together hinder the repair in the cartilage injuries and cause the osteochondral repair tissue to degenerate. Perhaps the novel tissue is too soft and does not withstand the high forces generated in the movements (Kisiday 2004, Levin 2005). Alternatively, the quantities of degrading enzymes are too much for the developing tissue, or the poor integration and impaired barrier fluid film in the superficial zone worsen the hydrostatic properties of the tissue and therefore make it mechanically unstable. Additionally, inflammatory responses may have an effect to the repair of the tissue (Namba 1998).

2.3.2 Osteoarthritis (OA) and cartilage degeneration

Once the tissue is injured it becomes more vulnerable to degeneration and development of posttraumatic osteoartritis (OA) (Brown 2006). The end result of posttraumatic OA resembles that of idiopathic primary OA, which is defined as a degenerative joint disease that affects all tissues of the joint. The disease is also known as degenerative arthritis or osteoarthrosis. The following features are linked to this condition: fibrillation and erosion of articular cartilage, osteophyte formation, subchondral bone sclerosis and synovial hyperplasia, fibrosis and inflammatory cell infiltration, although occurrence of these differs among patients (Buckwalter 1998a). At the molecular level, increased amounts of

degradative enzymes (Tchetverikov 2005), cartilage degradation products (Lohmander 2003), together with increased synthesis of ECM and inflammatory responses, can be observed (Dieppe 2005). The expression of type I collagen has been shown to increase in the osteoarthritic cartilage at the mRNA levels (Gebhard 2003, Martin 2001), and it is thought to indicate the tendency of the chondrocytes to repair cartilage injuries with fibrocartilaginous tissue. The pathogenesis of the OA is not fully understood, but once the degeneration has been initiated, it can progressively destroy the cartilage and weaken the function of the joint. Musculoskeletal disorders are one the most common causes to visit a physician, and the prevalence of knee OA is 6% in men and 8% in women in Finland according to a large survey (Arokoski 2007). At the moment there are no repair methods for OA, which would prevent the degeneration to proceed and restore lost cartilage, only palliative treatments (Dieppe 2005). Patients with posttraumatic OA are usually present with the disorder at a younger age. This further emphasizes the urge to generate cartilage tissue repair methods to prevent the development of OA after injury.

2.3.3 Methods of repair in cartilage injury

The earliest methods used to alleviate the pain and gain better functionality after cartilage injury included shaving and debridement of damaged cartilage. However, the relief in this case is only temporary, as the shaving does not stimulate repair. On the contrary, it can cause increased fibrillation and necrosis in the adjacent cartilage (Hunziker 2002, Kim 1991, Newman 1998). However, because the cleaning of flaps of fibrillated or injured cartilage can prevent mechanical problems as locking of the joints and further promote pain relief, this method is still used, when necessary (Gomoll 2010). Since osteochondral defects have the ability to repair at least to some extent, different surgical techniques have been developed where subchondral bone is surgically penetrated in lesions, which were originally chondral only in nature. These methods include drilling of multiple holes or creation of microfractures with an awl in the lesion site (Mithoefer 2005). The downside of these methods is the degeneration and deterioration of the repair tissue over time, a similar response detected in the spontaneous repair of osteochondrol defects (Hunziker 2002).

More drastic therapeutic interventions are those utilizing tissue or cellular transplantion to the defect site. Tissue transplantation techniques include transplantation of periosteal tissue or a graft containing bone and cartilage, called an *osteochondral* transplant (also known as mosaicplasty)

(Hangody 1998). The periosteal or perichondrial tissue potentially contains cells, which are capable of chondrogenic (and osteogenic) activity and, therefore, be able to promote the production of hyaline cartilage after the periosteal flap is sutured or glued on top of the cartilage defect in a surcigal procedure (Hunziker 2002, Newman 1998). However, the repair quality has not been satisfactory when using the perichondrial graft: deficient filling of the lesions, calcification of the cartilage and overgrowth has been reported, although symptomatic relief from pain is generally good (Bouwmeester 1997). In osteochondral transplantation a plug containing cartilage and bone is isolated from the less-weight bearing area of the joint and transplanted into the defected cartilage site (Hangody 1998). The downside of this procedure is obvious; it requires removal of osteochondral tissue from healthy cartilage sites and causes further destruction of the joint (Hunziker 2002). Additionally, the integration of the transplanted graft has been shown to be problematic and the quality of the repair tissue deteriorates over time in the patients (Bentley 2003). Damage to the healthy tissue in the patient can be avoided, if fresh osteochondral allograft transplantation (OAT) technique is used. With the OAT technique it is also possible to use tissue from similar, collateral location from an organ donor. The osteochondral allograft transtplantation have shown good results (Gross 2005), but the disadvantages include high costs, and troubles in the cell viability during transportation and surgery, as well as difficulties in finding suitable donor cadavers.

A more advanced tissue transplantation procedure is the autologous chondrocyte transplantation (ACT) method. In the ACT cartilage is first harvested from the less-weight bearing joint area. The chondrocytes are then isolated and multiplied in cell culture conditions prior to the second operation, in which the isolated cells are transplanted into the cartilage defect site underneath a periosteal or collagen flap (Brittberg 1994, Gomoll 2010). This method takes advantage of the chondrocytes' capacity to multiply and continue the production of ECM when they are released from their native ECM. Relief of pain and improved movements, accompanied with arthroscopic findings of hyaline cartilage resembling tissue, were reported in a nine year follow-up after ACT (Peterson 2000). The clinical outcome after ACT has been compared to microfracture by Saris et al (Saris 2009) and Knutsen et al (Knutsen 2007). In the study by Knutsen et al, both methods provided satisfactory results in 77% of the patients, and there were no significant differences in the clinical and radiographic results between the two treatment groups, and no correlation between the histological findings and the clinical outcome after five years (Knutsen 2007). However, when ACT was performed

using specially selected chondrocytes and characterized chondrocyte implantation (CCI), the results showed greater improvement in the CCI group of patients than in the microfracture group after 3 years follow up (Saris 2009). Furthermore subchondral bone reaction was better in CCI patients and it was concluded that chondrocyte quality and also time to treatment were shown to affect outcome. (Saris 2009). It has also been noted that the site and size of the lesion, the existence of other impairments of the joint and the age of the patient affect the outcome of the ACT (Knutsen 2007, Peterson 2000).

In the case of advanced OA or severe chondral or osteochondral defects, the last option for recovering functions is total joint arthroplasty, if other treatment methods have not been useful. Total joint replacement alleviates the pain and helps to gain better motility, but because the materials tend to wear off, especially in active patients, it is not recommended for younger patients. (Carr 2012).

2.4 TISSUE ENGINEERING AND BIOMATERIALS

The term tissue engineering (TE) covers a vast field of research and medical practices where tissues or organs are attempted to regenerate by delivering implanted cells, scaffolds, DNA, and/or proteins during surgical procedures in order to restore tissue morphology and function. This field emerged at the end of 1990's, and it uses a multidisciplinary approach, combining knowledge of cell and developmental biology, biomechanics, biomedical engineering, as well as material and transplantation sciences. (Getgood 2009). The current approaches in articular cartilage TE can be categorized into three technical routines where A) cells are combined with biomaterials and subsequently transplanted, B) cells are combined with biomaterials and pre-cultivated prior to transplantation, or C) biomaterial without cells is implanted (Figure 4).

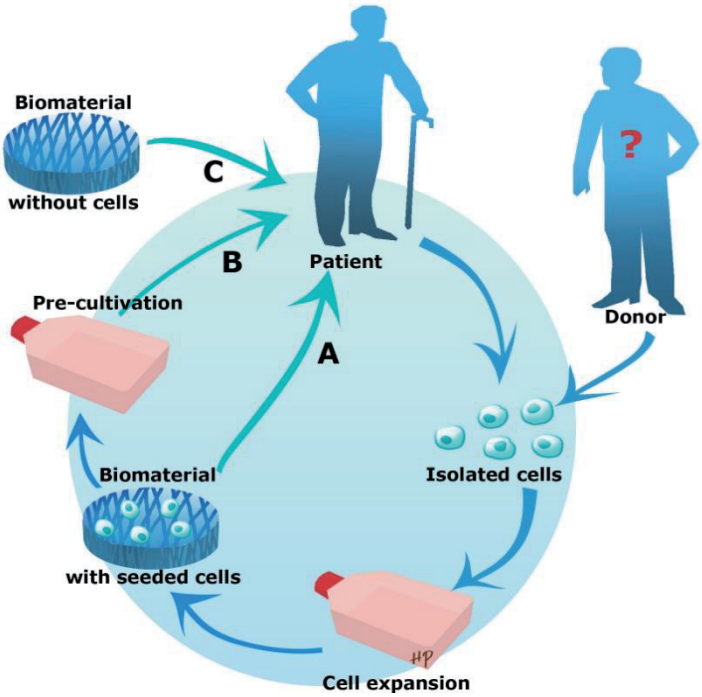

Figure 4. Principles of cartilage TE. First the autologous or donor cells are isolated and multiplied in cell cultures. The regeneration techniques include the following: A) cells are combined with biomaterial and transplanted straight after, B) cells and biomaterials are pre-cultivated in cell culture or bioreactor prior to transplantation, or C) acellular biomaterials are used.

2.4.1 Cartilage regeneration

It is apparent that the adult cartilage lacks functional mechanism to produce mechanically stable tissue and the spontaneous repair produces fibrous tissue prone to degeneration (Buckwalter 1998a). However, during embryonic development cartilage exhibits fast growth and also capability to regenerate (Goldring 2006), which was shown in a study where superficial cartilage defects created in fetal lambs and were left to heal *in utero* producing completely healed tissue after 28 days (Namba 1998). These findings suggest that articular cartilage regeneration might require an environment that mimicks the fetal events, and if possible, trying to prevent the inefficient spontaneous repair process (Caplan 2003). This task is not easy to conquer, as the embryonic development is a complex process largely unknown and the factors that determine wheter the cartilage develops into permanent type or transient to undergo hypertrophy, are also not understood. Therefore, with the current methods the *regeneration* of articular cartilage might be unreasonable task, and instead the realistic goals for cartilage TE might be to produce a *repair* tissue that withstands for a prolonged time and provides at least satisfactory functioning of the joints (Reinholz 2004).

2.4.2 Cell sources for cartilage tissue engineering

Different cell sources have been tested for articular cartilage TE. The optimal cells to be implanted or transplanted would be capable of producing new ECM similar to the native articular cartilage, and to be able to withstand the harsh circumstances that are present in the cartilage- the low oxygen and nutrients levels and high mechanical loads. The strict requirements narrow the candidates to either chondrocytes or undifferentiated stem cells.

Autologous chondrocytes are the earliest cell type that has been used for TE purposes in humans (Brittberg 1994). The chondrocytes would be the natural option as a cell source, as they are already programmed to produce cartilage ECM and suitable for the environmental challenges present in the organ. The drawbacks to this route are the associated dedifferentation during monolayer expansion (Brodkin 2004), and the tendency to produce fibrous repair tissue (Hunziker 2002). The chondrocytes can be isolated from the less-weight bearing cartilage of the patient (autologous chondrocytes), but then the disadvantage is the destruction of otherwise healthy tissue. The use of donor, allogeneic cells could be another option, since the studies of osteochondral allografting have shown that immunorejection is not a problem (Williams 2007).

Stem cells are cells with multipotent differentiation capacity. There are number of stem cell sources available, and adult mesenchymal stem cells

(MSC) have been especially investigated for cartilage TE (Getgood 2009). In adults, the MSCs are present in different tissues as a progenitor cell reserve populations. The MSCs in their undifferentiated stage will need instructions in the form of growth factors, cytokines and other environmental cues (load, suitable ECM and others) to start and continue the progress of differentiation (Caplan 2003). They can be harvested and isolated from adult bone marrow, adipose tissue, muscle, periosteum or synovium (Hunziker 2002), and then manipulated in cell culture conditions using specific growth factors to differentiate them into chondrocytic cells (Worster 2001) or directly transplant MSCs without differentation. A follow-up of 227 patients who underwent autologous bone marrow derived MSC therapy showed no neoplastic complications (Centeno 2010, Pelttari 2008). However, other studies are raising question about the tendency to undergo hypertrophy and development of vascularization in the MSC – derived chondrocytes *in vivo* (Pelttari 2008). Therefore, the use of MSCs as progenitor cells for cartilage restoration still needs further development.

There is also a vast field of ongoing research on optimization of cell culture conditions, for example to overcome the chondrocytes tendency to dedifferentiate during cultivation. It has been shown that the chondrocytes grown in monolayers lose their phenotype after longer culture periods (Brodkin 2004). Therefore, it has been investigated whether this adverse event could be avoided by manipulating the cell culture conditions, such as different oxygen level conditions (Murphy 2004), growth factors combinations (Chaipinyo 2002, Tew 2008), mechanical stimuli (Smith 2004), or three-dimensional scaffolds (Frondoza 1996) during cultivation. The use of bioreactors to cultivate cells alone or cells combined with biomaterials has gained much attention. The use of a bioreactor allows the tissue or cells to be exposed to mechanical stimuli, and improves the exchange of nutrients and waste products. (Risbud 2002). Bioreactors have been shown to increase the production of ECM in chondrocytes cultivated in a biomaterial, in comparison to static cultivation (Vunjak-Novakovic 1999). Other researchers have demonstrated increased stiffness, GAG and collagen content in chondrocyte-seeded hydrogel constructs cultivated in a TE bioreactor that applies daily cyclic compression. (Mauck 2000).

2.4.3 Biomaterials as scaffolds

Biomaterials are biocompatible materials that can be transplanted into human body without adverse effects. The materials are usually biodegradable and they offer initial support to chondrocytes in cartilage

TE. An optimal degradation process would proceed at the same pace new ECM is produced to replace the biomaterial (Caplan 2003). Biomaterials can be used as a scaffold for the chondrocytes or MSCs to promote the filling of the lesion, cell attachment, growth and differentiation in cartilage repair. There are several requirements for a good biomaterial: 1) the material and its degradation products need to be safe and non-toxic, 2) material should allow cell growth, exchange of oxygen, nutrients and waste products, and 3) material should provide mechanical support for the cells and help to fill the lesion site, and 4) material should offer attachment sites for ECM-receptors, that promote cell growth and prevent apoptosis (Getgood 2009).

Some biomaterials are used without cells (Gibson 2006, Steinwachs 2008), and the repair cells are then supposed to migrate into it from elsewhere. For example, cells from subchondral bone are exposed using the microfracture technique, and thay can inhabit the scaffold after transplantation (Benthien 2010, Steinwachs 2008). However, several applications take advantage of combining cells and biomaterials in order to grow new cartilage (Sittinger 2004).

There is no consensus amongst researchers about the optimal mechanical properties of the biomaterial for cartilage TE. Whether the materials should be hard for maximum support, or soft to allow cell seeding and growth, remains an open question. Some experts consider that the biomaterial should possess similar mechanical properties as adult articular cartilage. However, because of the special nature of this tissue this is not easily achieved and, by far, no such materials exist. The mechanical properties of the biomaterial are further complication. A hard material would provide better mechanical support, but this could lead to problems in cell seeding or submersion of the material into the subchondral bone (Pulliainen 2007), or detachment of the transplants (Vasara 2004). On the other hand, soft biomaterials such as hydrogels do not provide much support, but allow even cell seeding and growth of the transplant (Sittinger 2004).

Biomaterials that have been tested *in vitro* or *in vivo*, or are currently used as a repair method, can be categorized into natural materials or synthetic polymers. They have been developed in different formulas: sponges, woven scaffolds, fleece-like fabrics, or hydrogels. Hydrogels are networks of polymers containing large amounts of water. Because of the vast field of biomaterials, only the most popular or promising materials are presented in Table 1. Good reviews of different biomaterials are available (Hunziker 2002, Getgood 2009). The synthetic materials can offer good possibilities to formulate the structure and shape of the scaffold, but they can cause foreign body cell reactions and poor cell-

adhesion (Chu 1995, Hunziker 2002). Natural materials, especially those that naturally occur in the cartilage ECM, can offer a more familiar environment for the chondrocytes, and most importantly they provide attachment sites for the ECM-receptors, such as integrins, and can therefore inhibit apoptosis (Hirsch 1997). Collagens, especially, facilitate cell adhesion, enhance the growth and differentiation of cells (Kleinman 1981) while HA can be chondroinductive and antiangiogenic (Caplan 2003). Therefore, the most intensively studied natural materials and those that already have techniques in clinical practice for human patients are collagens in different formulas (Bartlett 2005, Frenkel 1997, Gibson 2006, Steinwachs 2008) and hyaluronan based matrices (Nehrer 2006, Solchaga 1999). The disadvantages of using the natural materials are the problems in shaping the matrix, the relatively soft form of their unprocessed state, and the availability of the materials. Most often the collagens are isolated from tendons or connective tissue from cattle or pigs, and they are usually type I or III collagens, not type II, which is present in articular cartilage (Kleinman 1981). Hyaluronan can be obtained from rooster combs or from specific bacterial cultivations (Widner 2005). The use of any animal-derived materials raises concerns about safety and poses a risk for allergic reactions. (Hunziker 2002)

Table 1. Examples of biomaterials that are currently tested for articular cartilage tissue engineering in in vitro studies, in vivo animal experiments, or in vivo clinical studies in humans.

Material	***Formula***	***Studied***	***Findings***	***Reference***
Synthetic				
Polylactide acid (PLA)	porous matrix	*in vivo,* rabbits	cartilaginous tissue after 6 weeks	(Chu 1995)
Natural				
Agarose	gel	*in vitro*	human chondrocytes maintained phenotype in long-term culture, but slowed down growth, retained ECM expression	(Kolettas 1995)
Alginate	beads	*in vitro*	bovine chondrocytes maintained phenotype in long-term culture	(Häuselmann 1994)
Cellulose, bacterial	sponge	*in vitro*	bovine chondrocytes were viable in short-term culture	(Svensson 2005)
Chitosan	coated surface	*in vitro*	human chondrocytes were viable in short-term culture (7 days)	(Lahiji 2000)

Collagen, type I, porcine	gel	*in vivo,* rabbits	hyaline-like repair tissue was detected after 6 months	(Kawamura 1998)
Collagen type I, CaRes-1S®, from rat tail	gel	*in vivo,* humans	complete filling of the lesion and improved clinical outcome after 24 months in nine patients	(Efe 2011)
Collagen type I, from rabbit skin	gel	*in vivo,* rabbits	hyaline-like repair tissue was detected after 24 weeks	(Wakitani 1989)
Collagen type I and III, porcine, called Chondro-Gide®	bilayered membrane	*in vivo,* rabbits	hyaline-like repair tissue was detected after 24 weeks	(Frenkel 1997)
Collagen type II, from bovine + polyethylene glycol (PEG)	gel	*in vivo,* rabbits	hyaline-like repair tissue was detected after 24 weeks	(Funayama 2008)
Collagen type I and III, procine, called MACI®	bilayered membrane	*in vivo,* humans	hyaline-like repair after 1 year in 36.4% patients, excellent clinical outcome in 19%	(Bartlett 2005)
Fibrin, called Tisseel®	adhesive gel	*in vivo,* rabbits	impaired the natural repair, prevents cell migration	(Brittberg 1997)
Hyaluronan (HA), HA benzyl-ester, called HYAFF® or Hyalograft C®	non-woven mesh	*in vivo,* humans	improved functional outcome 3 years post-operatively	(Nehrer 2006)

2.4.4 Assessing the repair methods and some future aspects

The assessment of the different biomaterials is rather difficult. All different matrices offer some good qualities and many studies report initially promising results *in vitro,* but unfortunately long-term repair of articular cartilage defects often faces the same end-result than the spontaneous repair: fibrillated and detoriated cartilage (Bartlett 2005). For analyzing the quality of the biomaterial, different *in vitro* and *in vivo* assessments are used. After the cells and biomaterials are cultivated *in vitro* or *in vivo,* the experiments are usually evaluated using different methods to analyze the cellularity or phenotype of the cells, the ECM production and structure, and mechanical properties of the products. In human studies the patients are also questioned about pain and ability to function.

The cultivation of isolated chondrocytes or MSCs is often needed to multiply the cells for cartilage TE, and the research done in cell culture systems *in vitro* has also provided many insights into the proliferation and differentiation of chondrocytes (Reinholz 2004). When novel biomaterials or TE methods are tested, the first step is usually *in vitro* cultivation under static conditions or in bioreactors to gain better insight on how the cells react. Primary chondrocytes, or chondrocytes passaged only a couple of times, are used in these experiments. The suitability of the biomaterial for chondrocyte cultivation is then detected using various microscopical and biochemical assays to assess cell viability, growth properties and ECM constituent production at both the RNA and protein levels.

If the preliminary *in vitro* studies confirm the usability of the TE method, then the material is usually tested *in vivo*, to further investigate how the material is reacting in living conditions. A heterotopic chondrogenesis model that uses immunocompromised nude mice has been developed to test the chondrogenic potential of the TE therapeutical methods *in vivo* (Dell'Accio 2001, Pelttari 2006, Silverman 1999, Solchaga 1999). The TE constructs are usually implanted under the back skin of the nude mouse. While the conditions in subcutaneous cultivation do not correspond to the circumstances present in joints, these studies offer an important first sight *in vivo* information, before more expensive animal studies with more effort are conducted. (Reinholz 2004).

Finally before the TE applications are accessible to be used on patients, the ability of the construct to produce repair tissue in actual defects needs to be proven in a larger animal model. Cartilage reconstruction has been studied in murine, lapine, canine, caprine, porcine, and equine models (Ahern 2009). Each model possesses advantages and disadvantages. The smaller species, such as mice, rats and rabbits are cheap to maintain, easy to handle and useful in the preliminary phase of studies. However, the smaller size of the joints and good spontaneous repair limit their usability. Larger animals, such as goats and minipigs provide larger joints, but still smaller than in humans, and their usage brings greater logistical, financial and ethical problems (Chu 2010). Althought none of the existing animal models are flawless, they are still needed in the future alongside with *in vitro* modeling, when developing surgical or therapeutical methods for cartilage TE.

The assessment of the repair quality in the animal models often includes description of the macroscopical observations of the repairs and surrounding tissue, microscopical and/or biochemical analyses of the ECM and chondrocytes, and biomechanical testing of the repaired tissue. The microscopical methods usually include use of various histologic dyes

for detecting different compartments of the tissue, and they provide illustrative data that can be used for quantification or scoring of the repair tissue quality. Scoring protocols are used to gain a more objective and comparable view of the repair quality (Mainil-Varlet 2003). For animal models, a scoring protocol developed by O'Driscoll and colleagues (O'Driscoll 1988) or Mankin and co-workers (Mankin 1971) have been used. In the O'Driscoll scoring protocol, usually several investigators individually grade blinded samples, investigating the cellular properties, nature of the predominant tissue, structural integrity, fibrillation and bonding to the adjacent cartilage.

In summary, research has really increased the knowledge about the potential of regenerative medicine in aiding articular cartilage repair. However, there are still many challenges for restoring the functionality of injured cartilage. Important goals for future studies include:

- determining the causes of repaired tissue degeneration and finding remedies to prevent it
- finding a method to promote intregration of the repaired tissue into the existing adjacent host tissue
- developing novel biomaterials that would promote healing in a clinically efficient way
- research the development of the multilayered structure of articular cartilage and finding a way to promote this in the repair tissue.

3 Aims of the study

The normally functioning articular cartilage offers smooth movements of the joints, but when injured it's spontaneous healing capacity is very limited. Currently no simple and good method is availlable that would guarantee regeneration and the subsequent healing of injured cartilage. Articular cartilage TE using biodegradable scaffolds for chondrocytes could help the restoration of functional tissue. The use of type II collagen as a scaffold material for chondrocytes has been extensively studied because it would offer a natural surrounding for the cells that could promote cell survival and phenotype maintenance. Type II collagen has been previously tested for TE with good results. The collagen material that has been previously tested was extracted from animals. If human collagen, manufactured with recombinant techniques, could be used instead, it would provide a reliable and animal component-free source of biomaterial.

The main aim of this thesis was to test a novel material; recombinant human type II collagen (rhCII) as a biomaterial for chondrocytes. The specific aims were the following:

1. **To explore different techniques and a variety of formulas of the rhCII, e.g., sponges, membranes and hydrogels, and their usability as a coating for other scaffolding materials (Studies I, II).**

2. **To determine and test the suitability of the rhCII material for chondrocyte cultivation *in vitro* (Studies I, II).**

3. **To investigate the feasibility of the developed method and formula of rhCII as a chondrocyte-seeded scaffold *in vivo* in terms of cellular, ECM and biomechanical properties (Studies III, IV).**

4 Materials and methods

This thesis consists of four independent studies (Figure 5), in which rhCII was tested for articular cartilage TE purposes using various methods to analyze the structural, biochemical and biomechanical properties of the constructs. In the preliminary studies, the rhCII was used as a membrane or sponge-like scaffold for chondrocytes (results published by Tiitu 2008). Secondly, the rhCII was used as a coating material for cellulose scaffolds and chondrocytes were cultivated in it *in vitro* (study I). From then on, the rhCII-material was tested as a gel-like scaffold for chondrocytes, first *in vitro* (study II), and then *in vivo* in a nude mouse model (study III), and in a cartilage defect repair study in rabbits (study IV), to investigate the suitability of the rhCII material for cartilage TE.

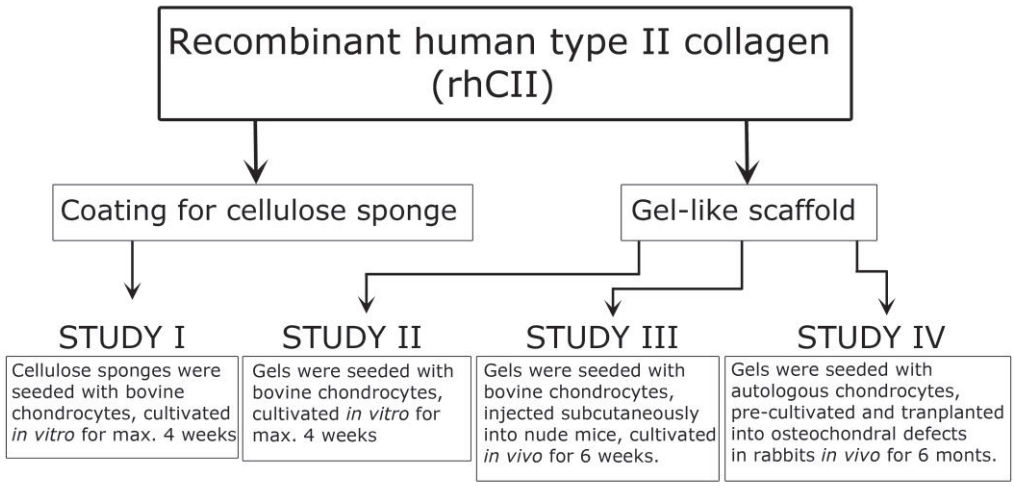

Figure 5. Outline of the thesis project.

4.1 BIOMATERIALS

4.1.1 Recombinant human type II collagen (rhCII) and other recombinant human (rh) collagens

Type II collagen has been previously tested and proven to be suitable for cartilage TE (Funayama 2008, Nehrer 1998). The rhCII-material was provided by a Finnish company- Fibrogen Europe Oy (Helsinki, Finland). They manufactured this material using recombinant DNA technology in yeast cultivations (*Pichia pastoris*). The product was then purified and dissolved in 10 mM HCl prior to further use. This process has been described by Myllyharju and colleagues (Myllyharju 2000). In brief, the manufacturing process utilizes gene transfer of cDNA for human procollagen α_1(II) into the yeast cells which coexpress recombinant prolyl hydroxylase (Nokelainen 2000, Olsen 2003). The recombinant prolyl hydroxylase catalyzes hydroxylation of prolines in the recombinant α-chains and has been shown to result in stable triple-helix formation of the α-chains that leads to the production of the procollagen molecule (Baez 2005, Olsen 2003). After harvesting the recombinant procollagen, it is treated with a protease that removes the N- and C-propeptides and allows formation of spontaneous aligned of collagen fibrils in neutral pH with close resemblance to native tissue-derived collagen (Olsen 2003).

The recombinant collagen was then used either as a sponge (Tiitu 2008), a membrane (Tiitu 2008), a coating for cellulose (study I) or a gel-like scaffold (studies II, III, IV). In preliminary studies, recombinant collagens I and III (Fibrogen Europe Oy, Helsinki, Finland) were also tested, and as a result it was decided to continue using the rhCII material for further studies (results presented in study III and by Tiitu 2008).

4.1.2 RhCII-coated cellulose scaffolds (study I)

Cellulose is a naturally occurring linear polysaccharide. It functions as a structural component in green plants and some bacteria. It is insoluble in water and degrades slowly when implanted in the human body (Martson 1999). Bacterial cellulose was recently introduced as potential scaffold for cartilage tissue engineering (Svensson 2005). In study I, viscose cellulose sponges® (Cellomeda, Turku, Finland) were used. The material has been developed to investigate wound healing, and these scaffolds are biocompatible and homogeneous sponge-like matrices (Pajulo 1996). The cellulose sponges were tested with and without rhCII-coating on the surface. For the coating procedure, the sponges were immersed in a solution of type II collagen (3.49 mg/ml) in 10 mM HCl for ten minutes and afterwards snap frozen in liquid nitrogen. After freezing the sponges

were freeze-dried under a vacuum, overnight, and subsequently cross-linked with a glutaraldehyde vapour treatment for 4 hours at 37°C. Unreacted aldehyde groups were blocked with 0.1 M glycine for 30 minutes and the sponges were then washed with deionised water, and further freeze-dried overnight (Chen 2003). Before cell seeding the sponges were sterilized with 70% ethanol, washed with sterile water and dried.

4.2 CHONDROCYTE ISOLATION, CULTURING AND VIABILITY ASSAYS

Freshly isolated bovine chondrocytes without precultivation were used in studies I, II and III. In the fourth study, autologous rabbit chondrocytes were used: rabbit cartilage was isolated during a surgical procedure, chondrocytes were isolated and then multiplied in cell cultures before using them in combination with rhCII material to make rhCII gels (study IV).

4.2.1 Chondrocyte isolation and cell culturing

Bovine articular cartilage was harvested from patellofemoral grooves of 18-month-old cows (Atria abattoir, Kuopio, Finland). Chondrocytes were isolated from cartilage slices in a two-step isolation protocol performed at 37°C and an atmosphere containng 5% CO_2. The cartilage was digested with 500 μg/ml of hyaluronidase (Sigma, St. Louis, MO, USA) in Dulbecco's modified medium (DMEM, Euroclone, Pero, Italy) for 30 minutes. In the second digestion step, DMEM containing 300 μg/ml of collagenase (Sigma) and 200 μg/ml of DNAase I (Sigma) was used overnight. Next day, the cells were washed with phosphate-buffered saline (PBS) and suspended in DMEM supplemented with 1% fetal calf serum (FCS, PAA, Linz, Austria), 2 mM L-glutamine (Sigma), 200 IU/ml of penicillin (Euroclone), 200 μg/ml of streptomycin (Euroclone), 2.5 μg/ml of fungizone (Sigma) and 50 μg/ml of ascorbic acid (Sigma).

Rabbit chondrocytes were obtained by harvesting a biopsy of cartilage from the margin of the femoral trochlea of the left knee joint. The surgical operations were performed under anesthesia (Domitor 0.35 ml/kg, Orion Pharma, Espoo, Finland and Ketaminol 0.6 ml/kg, Intervet International B.V., Boxmeer, the Netherlands). Subsequently, the cartilage biopsies (10-20 mg/each isolation) were washed with sterile phosphate-buffered saline (PBS). Cartilage was digested with 120 units/ml of collagenase (Sigma) in

DMEM at 37°C and 7% CO_2 overnight. The next day, the isolated cells were washed with PBS and suspended in supplemented DMEM. After enzymatic digestion the cells were expanded in culture dishes in an incubator at 37°C and 7% CO_2, until the desired amount of cells was abtained in approximately 2-3 weeks. The expanded cells were trypsinized and used for making the hydrogels before transplantation into cartilage defects.

4. 3 THE USE OF RHCII-MATERIAL AS A COATING OR GEL-LIKE SCAFFOLD

4.3.1 Cell seeding into rhCII-coated cellulose sponges (study I)

Freshly isolated bovine chondrocytes were seeded into the sterile cellulose scaffolds by adding medium containing 1.225×10^6 cells per scaffold. For other experiments, the cells were cultured in scaffolds for 1, 2, 3 and 4 weeks in 37° and 5% CO_2. The viability of the chondrocytes was tested using the viability assay described in section 4.7.1.

4.3.2 Preparation of rhCII hydrogels (studies II, III, IV)

The cell-seeded collagen gels were made using rhCII solution (3.7 mg/ml dissolved in 10 mM HCl) with bovine (study II, III) or autologous rabbit chondrocytes (study IV). Collagen is soluble in acidic solution, but when the collagen solution is neutralized into pH of physiological level in 37°C degrees, the collagen molecules spontaneously arrange into their native helical form (Elsdale 1972). The suitable concentration of chondrocytes and rhCII-solution was first determined in preliminary studies *in vitro* (data not shown). It was noticed, that when the collagen solution in 10 mM HCl is diluted 1:1 to medium, the solution was neutralized (confirmed with pH meter).

4.3.3 Chondrocyte seeding into rhCII hydrogels (studies II, III, IV)

Collagen gels were individually prepared by gently mixing the rhCII containing solution and the medium containing chondrocytes at a 1:1 volume ratio in sterile microcentrifuge tubes. A relatively high cell number was used to increase the structural integrity and durability of the gel. The number of cells was 1×10^6 per 100 µl of medium, the total concentration in the intitial solution then being 500 000 cells in one hydrogel. Cell-free controls were made in a similar fashion, but without adding cells. Gels were incubated at 37°C and 5% CO_2 for 1 hour to allow gelation of the collagen. After incubation, the gels were centrifuged at 13 000 rpm for 2 minutes, and excess liquid was removed. The remaining

part (the soft collagen gel with or without the chondrocytes) was then transferred to carrier plates on top of cell culture dish (studies II, IV), or transplanted into nude mice (study III). The carrier plates, similar to those used in histological sample preparation, were used to prevent the hydrogels becoming attached to the bottom of the dish and to provide support to the hydrogels. The outcome was that hydrogels were floating in the supplemented growth medium inside the carriers during the cultivation.

4.3.4 Cultivation *in vitro* (studies I, II)

Chondrocyte growth medium consisted of DMEM supplemented with 10% fetal calf serum (PAA, Linz, Austria), 50 μg/ml ascorbic acid (Sigma), 2 mM L-glutamine (PAA), 20 IU/ml of penicillin (Euroclone), and 200 μg/ml of streptomycin sulphate (Euroclone). The growth medium was added to the dishes, and half of it was replenished with fresh medium twice a week. The cellulose sponges were floating in the growth medium in cell culture dishes, and the rhCII-gels were cultivated inside the carrier plates positioned in the cell culture dishes.

4.3.5 Macroscopic evaluation of the rhCII hydrogels (study I)

The macroscopic appearance of the rhCII hydrogels was examined during the culture time. Typically the collagen gels tend to contract during culture (Galois 2006). Therefore, the gels were photographed at different time points to estimate the extent of gel contraction.

4.4 ANIMAL EXPERIMENTS

The study design of the animal experiments was approved by the Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government.

4.4.1 Nude mouse model (study III)

Immune-deficient nude mice have been used for various purposes in cartilage research. They were first used when chondrocytes from rabbits and dogs were injected subcutaneously (Lipman 1983). Since then, nude mice have become a commonly used animal model for designing matrices for cartilage reconstruction (Munirah 2008, Solchaga 1999), e.g.,

to testing *in vivo* chondrogenic cell differentiation and cartilage formation (Dell'Accio 2001, Enomoto-Iwamoto 2000).

In our study nude mice were anaesthesized with 2-4% isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Isofluran Baxter, Baxter, Lessines, Belgium) before injections. Each mouse (n=24) was treated with three subcutaneous injections (Figure 8A): 1) cell-free rhCII gels (rhCII-gel); 2) bovine chondrocytes in rhCII gel (rhCII-cell); and 3) bovine chondrocytes in growth medium (Med-cell). The volume of injections was 400 μl that contained 6 million cells. The injection sites were located in the back of the mice in a triangular pattern (Figure 8A). No major scar formation or skin abnormalities were detected in the injection sites, and the animals behaved normally. After 6 weeks, the animals were euthanized with CO_2, and the constructs and blood samples were collected for further analyses. Specimens (24 in each group) were collected from these triplicate injections and divided for further analyses. The total number of individual specimens was 7-12 in the analysis. Samples from rhCII-gel injections could not be used for further analyses, since no tissue formation was observed in the implantation sites.

4.4.2 Blood samples from nude mice (study III)

Blood samples were collected from the mice at the time of death. The values of leukocytes, erythrocytes, hemoglobin and thrombocytes were analyzed with an automated hematology analyzer (Cell Dyn 3500R, Abbott Laboratories, Abbott Park, IL), which has been shown to be a reliable and accurate instrument for murine blood analysis (Kieffer 1999). Blood of the animals injected with rhCII (n=12) was compared to that of the non-injected control animals of the same age (n=10).

4.4.3 Rabbit model for cartilage injury (study IV)

Rabbits have been widely used as an osteochondral defect model for cartilage repair (Brittberg 1997, Christensen 2012, Funayama 2008, Sah 1997, Willers 2005). To study the feasability of using the combination of rhCII and autologous chondrocytes to repair cartilage injuries, osteochondral defects (4 mm in diameter and 3 mm in depth) were created into rabbits' femoral trochlea (patellar groove) by drilling. A total of 15 mature female New Zealand white rabbits (age 9 months) were used for this experiment. Rabbits were randomly divided into two groups and defects to the articular cartilage were created, and treated as follows: 1) defects were repaired with precultivated rhCII hydrogels and previously expanded autologous chondrocytes (rhCII-repair, n=8); and 2)

defects were left empty (spontaneous repair, n=7). The repair results after 6 months were compared with intact patellar groove cartilage samples (n=7) taken from the contralateral femurs of the spontaneous repair group animals.

The rabbits in the rhCII-group underwent two surgerical operations; in the first operation a biopsy of cartilage for chondrocyte isolation was harvested from the margin of the femoral trochlea of the left knee joint and multiplied as described in chapter 4.2.1. The expanded cells were trypsinized and used for preparation of the gels as follows: 300 µl of DMEM containing 3 million chondrocytes was combined with 300 µl of rhCII-solution. Excess liquid was removed by centrifuging after 1 hour of gelling at 37°C.

The rhCII cell constructs were cultivated in cell culture conditions for 2 weeks prior to the next surgical operation to minimize the influence of early stage contraction in the gels. The surcigal operations were performed under anesthesia (Domitor 0.35 ml/kg, Orion Pharma, Espoo, Finland and Ketaminol 0.6 ml/kg, Intervet International B.V., Boxmeer, the Netherlands). In the second surgical operation, an osteochondral lesion (4 mm in diameter, 3 mm in depth) was drilled into the right femoral trochlea (patellar groove) (n=8) and filled with the pre-cultivated rhCII-cell construct. Before implantation, the gel grafts were punched with a 4 mm biopsy punch (Kai Industries, Oyana, Gifu, Japan). The precise size of the gel grafts allowed tight fitting of the implant into the defect without any additional fixation. For the spontaneous repair group, similar lesions were created (n=7) and left empty. At the end of the experiment, the contralateral knees (n=7) from this spontaneous repair group were used for harvesting intact tissue from patellar groove as a control reference. After 6 months, the animals were killed and the knee joints (distal end of femurs) were stored in a freezer (-20°C) in PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA; Merck KGaA, Darmstadt, Germany), 5mM benzamide-HCl (Sigma St. Louis, MO, USA) and 5µg/l phenylmethylsulfonylfluoride (Sigma) as inhibitors of proteolytic enzymes, until further analysis. Micro-computed tomography (µCT) and biomechanical analyses were performed before histological fixation of the samples for tissue imaging analysis.

4.5 METHODS FOR ANALYZING THE RESULTS

During the thesis project, several methods were used to evaluate the properties of the rhCII material as a coating agent, and as a gel-like

scaffold for chondrocytes. The used methods for analyzing the results and outcome of the studies are listed in the Table 2 below. Specific details are presented in the following chapters.

Table 2. Methods used to study rhCII material as a scaffold for chondrocytes in the original publications.

Investigated property	**Study I** ***in vitro***	**Study II** ***in vitro***	**Study III** ***in vivo***	**Study IV** ***in vivo***
Cell viability				
viability assay with confocal microscope	X	X	X	X
Cellularity				
hematoxylin staining			X	
Cell morphology				
SEM	X			
Proteoglycans (PGs)				
safranin O histology		X	X	X
toluidine blue histology			X	X
PG content with FTIR				X
structural PG analysis with electrophoresis			X	
PG contents with uronic acid analysis		X		
aggrecan expression by RT-PCR		X		
Collagens				
Col2a1 expression by RT-PCR		X		
type II (COL2A1) immunostaining		X	X	X
type I (COL1A1) immunostaining			X	X
type X (COL10A1) immunostaining			X	
collagen content with FTIR			X	
collagen parallellsim with e-POL			X	X
fibril orientation with e-POL				X
Bone formation and quality				
alkaline phosphatase histology			X	
Masson’s trichrome histology			X	X

Micro-computed tomography				X
Mechanical properties				
mechanical testing	X		X	X

4.6 GENE EXPRESSION LEVELS IN CELL CULTURES (STUDY II)

4.6.1 Gene expression analysis by quantitative RT-PCR

Total RNA was isolated from the bovine chondrocytes grown in recombinant human type II collagen gels using Eurozol reagent to disrupt the cells (Euroclone). After extraction, 1 μg of total RNA was treated with 1 μl of 2 U/μl of Turbo DNA-free DNAase according to the manufacturer's instructions (Ambion, Houston, TX, USA) . 1 μg of DNA-free RNA samples were then reverse-transcribed into cDNA in a reaction mixture containing 10 mM dNTPs, 25 mM MgCl2, 100 mM dithiothreitol, random DNA hexamer, RNase inhibitor and Multiscribe Reverse Transcriptase in 5 x Reverse Transcriptase (RT) buffer (PE Biosystems, Foster City, USA). Expression levels of aggrecan, procollagen α1(II) and GAPDH mRNA were analyzed using quantitative real-time PCR (Mx3000P, Stratagene, La Jolla, USA). The primers used for PCR are listed in Table 3. The PCR analysis was performed in a 25 μl reaction volume containing 40 ng of cDNA, 2 x SYBR Green Master Mix and 100 to 600 nM of each primer. The amplification protocol included a first cycle at 95°C for 10 minutes, then 40 cycles at 95°C for 30 seconds, 59-60°C for 1 minute, and 72°C for 1 minute. The efficiency of the amplification was between 90-110% (calculated using the software by Stratagene). The melting curve analysis was done after the amplification to ensure that only one end-product had been synthesized. The PCR products were also analyzed by electrophoresis to estimate that the product was of the correct size. The cDNA bands were excised from the gels, purified and then sequenced to confirm the presence of the correct amplified product. Sequencing was performed by personnel from A.I Virtanen Institute for Molecular Sciences (University of Kuopio, Finland) using the primers from Table 3. The obtained sequences were verified to be correct using the Basic Local Allignment Tool (blast.ncbi.nlm.nih.gov). The results of the PCR analysis were normalized using bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference transcript.

Table 3. Primer sequences for the real-time PCR assays.

Gene	***Primers (5'-3')***	***Product size (bp)***	***Reference***
Col2α1	TGAGAGAGGGGTTGTTGGAC AGGTTCACCCTTCACACCTG	133	(Galois 2006)
Aggrecan	CACTGTTACCGCCACTTCCC GACATCGTTCCACTCGCCCT	303	(Bosnakovski 2006)
GAPDH	TTCAACGGCACAGTCAAG ACATACTCAGCACCAGCATCAC	119	RTPrimer DataBase ID: 3379 (http://medgen.ugent.be/rtprimerdb)

4.7 MICROSCOPY

4.7.1 Cell viability assay for chondrocytes

The viability of chondrocytes cultivated in the constructs was evaluated using a combination of two fluorescent probes. The specimens were incubated for 5 minutes in a solution containing 60 μM concentration of cell membrane-impermeable DNA-binding dye, propidium iodide (Sigma, St. Louis, MO, USA and 10 μM concentration of cell-permeable fluorescein diacetate (Fluka, Buchs, Switzerland) in PBS. After washing with PBS, the samples were viewed with a confocal scanner (PerkinElmer Life Sciences, Wallac-LSR, Oxford, UK) on a Nikon Eclipse TE300 microscope, using the wavelengths 488/510 nm (excitation) and 525/550 nm (emission) for fluorescein, and 568/610 nm (excitation) and 607/645 nm (emission) for propidium iodide.

4.7.2 Scanning electron microscopy (SEM) (study I)

Cell morphology and expansion in the cellulose scaffolds was analysed using SEM. After the 4 weeks culture period, the scaffolds were fixed in 2.5% glutaraldehyde dissolved in 0.1M sodium cacodylate buffer (pH 7.4). They were subsequently dehydrated step wise through an ethanol gradient (50, 70, 80, 90 and 100% ethanol) and finally with hexamethyl-disilazane. The scaffolds were coated with gold and viewed with Philips XL30 ESEM TMP (FEI Company/Oy Philips Ab, Brno, Czech Republic).

4.7.3 Preparing the samples for histology and imaging (studies II, III, IV)

The cellulose scaffolds proved impossible to process into histological samples, so no histology could be done with the cellulose cultivations. The *in vitro* cultivated rhCII gel constructs were initially very soft, and after 4 weeks of cultivation they were firmer, but still difficult to process for histology using the conventional methods. Therefore, the *in vitro* cultivated gel samples were fixed overnight in an aquoes solution containing 4% formaldehyde and 0.5% Safranin O (Fisher, New Jersey, NY, USA) to obtain better preservation of proteoglycans in the samples (Kiraly 1996) (study II). For the subcutaneously cultivated samples collected from nude mice, the constructs were harder and could be processed using the traditional overnight fixation in a 4% formaldehyde solution (study III). The tissue repair samples from the rabbits contained bone and cartilage dissected from the operated joint, and therefore decalcification of the sample was required in order to soften the bone (study IV). The rabbit knee joint samples were first sawed into small, approximately 1 cm x 0.5 cm x 0.5 cm pieces that contained bone and cartilage from the lesion area or equivalent intact tissue. The specimens were then fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h. The formaldehyde-fixed samples then underwent a decalcification process in 10% EDTA (Merck KGaA, Darmstadt, Germany) that took place for several weeks.

Subsequently, after fixation all the samples were embedded in Tissue-Tek III embedding wax (Sakura Finetek Europe, Zoeterwoude, Netherlands) and cut into 3 μm or 5 μm thick sections (in the rabbit samples perpendicular to the joint surface) for the histology, immunohistology, enhanced polarized light microscopy and Fourier-transform infrared imaging spectroscopy.

4.7.4 Histological stainings and analysis

Proteoglycans were stained with cationic stains toluidine blue or safranin O, and bone with Masson's trichrome staining. The possible hypertrophy indicating endochondral ossification of the chondrocytes was detected using alkaline phospatase (ALP) staining in the study III. During the endochondral ossification the chondrocytes become hypertrophic and start to secrete ALP, therefore it can be used as a marker for hypertrophic chondrocytes. ALP activity was demonstrated by 2 hr incubation in ALP substrate solution (0.2 mg/ml Naphtol AS-MX and 0.4 mg/ml Fast Red TR in 100 mM Tris-maleate buffer, pH 9.2) after overnight incubation with MgCl (1% in Tris-maleate buffer, pH 9.2) (Miao 2002). Mouse embryo and

bovine cartilage were used as positive and negative controls, respectively.

The modified O'Driscoll score (maximum score 30) for cartilage repair was used to evaluate the repair quality from the safranin O stained sections (O'Driscoll 1988) in the rabbit study (study IV). Three observers scored the blinded samples independently, and the scores were averaged and rounded off to the nearest integer. In this histological scoring a variety of cellular and ECM properties were analyzed and evaluated using numerical values.

4.7.5 Immunohistochemical staining

Type II collagen was detected using the mouse monoclonal antibody E8 (Holmdahl 1986) (studies II, III, IV). This antibody is directed against chicken type II collagen, but cross-reacts with several mammalian species, such as murine type II collagens (Sahlman 2004). The primary antibody was applied for an overnight incubation (1:500), after 1 h hyaluronidase treatment (Sigma, 2mg/ml in PBS, pH 5). It was detected using the Envision Plus kit (Dako, Glostrup, Denmark). Anti-type I collagen (Abcam, Cambridge, UK) was used with an overnight incubation (1:100) at 4°C, after 20 min in 0.1% pepsin (Sigma, St. Louis, MO, USA) (in 0.5M acetic acid) (study III) or 30 min in 1mg/ml pronase (Callbiochem, San Diego, USA) (study IV), with a subsequent 1 h hyaluronidase treatment at 37°C (studies III, IV). Type X collagen was immunostained with mouse monoclonal antibody X53 (Quartett, Berlin, Germany) with an overnight incubation (1:500) at 4°C (Eerola 1998) after pepsin and hyaluronidase treatments, as described above. For type I and X collagens the Envision+ System-HRP (Dako, Glostrup, Denmark) was used for staining. Positive and negative controls (rabbit neonatal or juvenile adult cartilage and bone) were used to confirm reliability of the tests and specificity of the antibodies, and negative controls without primary antibody (1% BSA in PBS) were included.

4.7.6 Enhanced polarized light microscopy

Sample sections (5 μm thick) were cut, dewaxed in xylene, and rehydrated as previously described (Hyttinen 2001). Tissue glycosaminoglycans (GAGs) were digested with bovine testicular hyaluronidase, type IV (Sigma, St. Louis, MO, USA) using 1000 units/ml in phosphate buffer for 18 hours at 37°C. The sections were left unstained, embedded in DPX mounting medium (Difco, East Molesey, UK), and examined with a computerized Leitz-Ortholux POL microscope

using a 5.7 μm pixel size. The degree of parallellism of the collagen network (as measured by the parallellism index) and the orientation angle of the collagen fibrils were determined (Rieppo 2008). The parallelism index (PI) indicates the anisotropy of the material, that is, whether the fibrils are running in the same direction or not. A higher PI (maximum value is 1) indicates that the majority of the collagen fibrils are running in the same direction at that given site.

4.7.7 Fourier-transform infrared imaging spectroscopy

5-μm-thick sections were prepared similarly to those used for polarized light microscopy (but without the digestion of GAGs), and transferred onto 2-mm-thick infrared-transparent ZnSe-windows. Fourier transform infrared imaging spectroscopy (FT-IRIS) was utilized to determine the amide I (1584-1720 cm-1) absorbance of the sections as a measure of collagen content, whereas the carbohydrate region (984-1140 cm-1) was used to estimate the proteoglycan content (Julkunen 2009). Measurements were done using a Perkin Elmer Spectrum Spotlight 300 FTIR imaging system (Perkin Elmer, Cambridge, UK) at a 4 cm-1 spectral resolution and with a pixel size of 25 μm.

4.8 BIOCHEMICAL ANALYSIS

The quantitative and structural analysis for proteoglycans using biochemical methods was done for the constructs. After measuring the wet weight of the samples, they were freeze-dried, and the dry weights of the samples were determined.

4.8.1 Quantitative analysis of proteoglycans (PGs)

For quantitative analysis, the freeze-dried and weighted samples were processed with papain digestion (study III). The papain digestion included incubation with 1 mg/ml concentration of papain (Sigma, St. Louis, MO, USA) in 150 mM sodium acetate including 50 mM Cys-HCl (Sigma) and 5 mM EDTA (Merck KGaA, Darmstadt, Germany), pH 6.5, for 3 hours at 60°C to digest the PGs. Afterwards the samples were boiled for 10 min to inactivate the enzyme. The uronic acid contents of the digests were quantitated from the ethanol-precipitated samples (Blumenkrantz 1973). The amounts of uronic acid were normalized to the

wet weights of the samples to compensate for the variation in the size of the sample(II).

4.8.2 Structural analysis of PGs

The structural analysis was conducted using electrophoresis. The specimens were extracted with 4 M guanidinium HCl dissolved in (Gu-HCl) 50 mM sodium acetate, pH 5.8, supplemented with 10 mM disodium EDTA (Merck), 5 mM benzaminide HCl and 100 mM 6-aminohexanoic acid for 48 hours. For agarose gel electrophoretic analysis of PG subpopulations, the samples were then precipitated with 75% ethanol. The precipitated samples (1 μg of uronic acid) were dissolved in 7 μl of sample buffer (1% SDS in 40 mM Tris-acetate and 1 mM sodium sulphate, pH 6.8), boiled for 5 min, and 7 μl of 60% sucrose and 0.05% bromphenol blue in sample buffer was added to each sample. The electrophoresis in 1.2% agarose was performed in 40 mM Tris-acetate and 1 mM sodium sulphate, pH 6.8, for 3 hours at 60-80 mA and 40 V. After electrophoresis, the gel was fixed for 2 hours in methanol:acetic acid:water (50:7:43, v:v:v) mixture, and stained with 0.02% toluidine blue in 3% acetic acid for 4 hours. Excessive stain was removed by washing in a 3% acetic acid solution, and the gel was scanned. Similarly treated bovine articular cartilage (Gu-HCl and papain exracted) and chondroitin sulphate C (Sigma) were used as a positive control and mouse skin PGs (Gu-HCl extracted) as a negative control.

4.9 MICRO-COMPUTED TOMOGRAPHY FOR ANALYSING SUBCHONDRAL BONE HEALING

4.9.1 Micro-computed tomography (μCT) analysis (study IV)

In order to measure and detect structural changes in the subchondral bone in the rabbits, the knee joint samples were scanned with a μCT scanner (Skyscan 1172 Skyscan, Aartselaar, Belgium). Tube voltage of 100 kV was applied with voxel size of 15 x 15 x 15 μm3. The μCT images were analyzed with the CT Analyzer software version 1.9.1.0 (SkyScan).

The 3D-analysis was performed from two separate, manually selected volumes of interest (VOI) for each sample. The VOI 1 (a cylinder with 4 mm diameter and 2 mm in height) was placed within the deep part of the healed osteochondral lesion, while VOI 2 (a cylinder with 4 mm diameter and 1 mm height) was placed below the lesion. Following parameters were calculated for these two VOIs: bone surface- to volume ratio

(BS/BV), bone volume fraction (BV/TV), trabecular bone pattern factor (TBPf), structural model index (SMI), trabecular thickness (TbTh), trabecular number (TbN) and trabecular separation (TbSp).

4.10 MECHANICAL TESTING

The mechanical properties of samples were tested using a computer-controlled high-resolution material testing device (Laasanen 2002) applying unconfined compression under displacement control (stress-relaxation test) (study I, III), or an indentation technique under load control (creep test) (study IV) (Julkunen 2009). In unconfined compression, the sample is placed between two smooth impervious platens and during compression the fluid can freely flow laterally. In the indentation test the sample is compressed with a plane-ended indenter that has smaller diameter than the sample. During the stress-relaxation test the specimen is first subjected to constant deformation (μm), and then during relaxation the decrease in the stress (Pa) over time is recorded. The creep test is performed applying constant stress (Pa) on the specimen and time-dependent deformation of the sample is recorded (μm). To minimize the effects of proteolytic enzymes, the samples were balanced in a PBS bath containing protease inhibitors during the measurements (Rieppo 2003).

The recorded data was then used to calculate Young's modulus at equilibrium, also called the equilibrium modulus (E), and dynamic modulus (E_{dyn}), also known as instant modulus. The equilibrium modulus characterizes the properties and sfiffness of cartilage after the fluid flow and deformation has stopped in the tissue. The dynamic modulus reflects the response of the tissue to cyclic deformation (Hayes 1972) and stiffness of the cartilage during loading. The exact details for measurements are explained in the original publications of the studies II, III and IV.

5 Results

5.1 PRELIMINARY STUDIES AND BIOMATERIAL SELECTION

In the preliminary studies rh collagens type I, II and III were tested. Based on the fact that type II collagen is the prominent collagen in the articular cartilage and on histological findings after 28 days of *in vitro* cultivations, the studies were carried on with rhCII (results presented in study III).

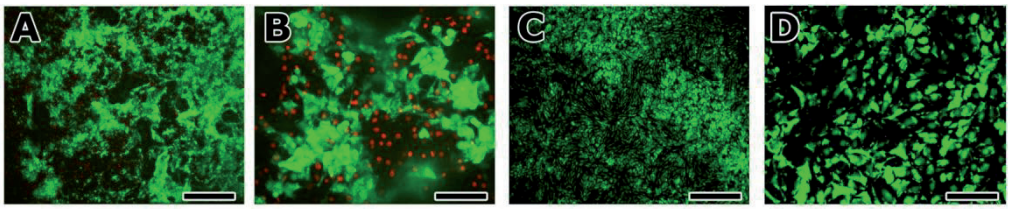

Figure 6. Confocal microscope images after 4 weeks of *in vitro* cultivation of bovine chondrocytes in rhCII sponges (A-B) and rhCII membranes (C-D). Scale bar = 500 µm (A,C), scale bar 100 µm (B,D). Live cells are green, and dead cells red.

Subsequently, the rhCII material was tested as a coating for cellulose scaffolds (Study I), as a membrane (Tiitu 2008), as a sponge (Tiitu 2008) and as a gel-like scaffold (also called hydrogel) (studies II, III, IV). The rhCII coating improved the chondrocytic phenotype preservation in the first study, but the cellulose material itself was not optimal as a scaffold material, and it was not further investigated. When chondrocytes were cultivated in the rhCII membranes, they dedifferentiated and become fibroblast-like, could not infiltrate inside the material and populated only the surface of the membrane (Tiitu 2008). Therefore, the membrane could not provide optimal conditions for the chondrocytes in the cartilage repair procedure. In the rhCII sponges the phenotype of the chondrocytes was better preserved, cell seeding was problematic and the cells did not reach the inner parts of the sponges. In addition, some areas contained dead cells when sponges were used as a scaffold for chondrocytes. In the

rhCII collagen hydrogels, however, the cells were evenly distributed and the chondrocytes maintained their round phenotype for 4 weeks. Consequently, the gel-like rhCII scaffold was selected for further studies and modifications.

5.2 CHONDROCYTE CULTURES *IN VITRO* (STUDIES I, II)

When rhCII was used as a coating material for the cellulose sponges, the suitability of the rhCII material for chondrocyte cultivations was confirmed, as the cells in rhCII-treated sponges better maintained their round morphology for 3 weeks, whereas the cells in the cellulose sponges appeared to be flatter (Figure 6A and B). Furthermore, cell viability was detected to be good in the chondrocytes grown in rhCII treated sponges (Figure 6C).

After these initially promising results, the remaining studies were focused on using rhCII-coated material as a gel-like scaffold (hydrogel). Cell viability remained good when the chondrocytes were cultivated in the rhCII gels *in vitro* (Figure 6D). It was noticed that the structure of the rhCII gels evolved during the *in vitro* cultivations. Initially the freshly fabricated rhCII hydrogels were very soft with jelly-like soft concistency that could be transferred by a pipette (Figure 7A). During the 4-week cultivation period of rhCII hydrogels and chondrocytes, the general appearance of the constructs changed into a clearly harder and compact material that could be balanced on a spatula (Figure 7B). However, the material was too soft for mechanical testing. Macroscopical evaluation of the gels showed that they also contracted during the *in vitro* cultivation period (Figures 7A and B). The contraction was most marked between cultivation weeks 1 and 2 and the total contraction was approximately 47% during the 4 week long cultivation time. The cultivation method of using carrier plates (Figure 7A and B) to keep the hydrogels floating and prevent them to attach the culture dish, was proven successful.

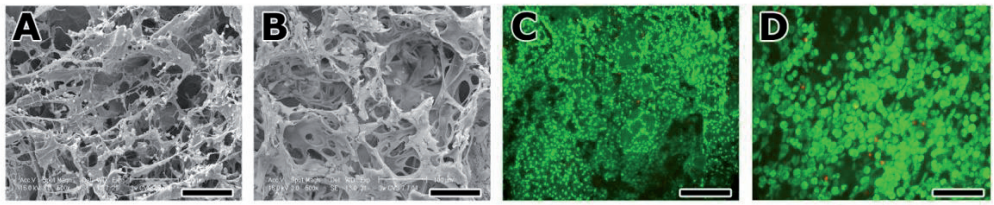

Figure 6. Scanning electron microscope (SEM) images after 4 weeks of *in vitro* cultivation of bovine chondrocytes in cellulose with rhCII coating (A) and without coating (B). A-B scale bar = 100 µm. Confocal microscope images after 4 weeks of *in vitro* cultivation of bovine chondrocytes in cellulose with rhCII coating, scale bar = 500 µm (C) and in higher magnification showing round chondrocytes, scal bar = 100 µm (D). Live cells are green, and dead cells red.

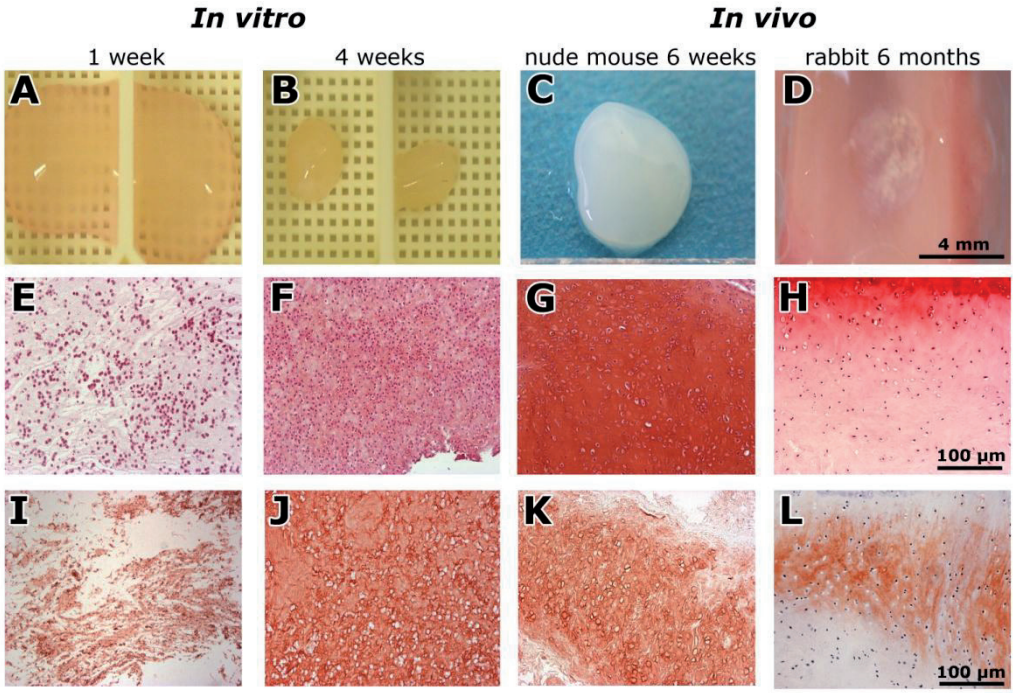

Figure 7. Compilation of findings in studies II, III and IV. Photographs from these studies in the first line show the gradual development of the rhCII constructs in the course of the studies from a soft gel consistency into firmer structure (A-D). In the second line, the amount of PGs showed increase indicated with safranin O staining (E-H). In the third line, the amount of type II collagen was also detected to increase as evaluated with anti-type II collagen staining (I-L). The tissue quality in the rabbit repair samples was not homogenous (H and L).

Histological analysis revealed that the chondrocytes were evenly distributed throughout the rhCII-hydrogels and the number of the cells increased during the culture periods of 1 day until 4 weeks (Figure 7E and F). The cells also started to produce ECM, which was shown as increased intensity of safranin O staining of the rhCII gels (Figure 7E and F). Type II collagen synthesis and secretion also increased during the cultivation period. This was indicated by the observation of a more intense staining of pericellular type II collagen around the chondrocytes (Figures 7I and J). The overall intensity of the type II collagen staining demonstrating increased density of type II collagen was also present. However it should be noted that this can partly result from the contraction of the gels.

The ECM production of the chondrocytes cultivated *in vitro* in the rhCII-hydrogels was further assessed using uronic acid analysis and RT-PCR. Since uronic acid is part of the GAG structure in the cartilage PGs, analysis of its content in the tissue gives an estimate of the PG content. The quantity of GAGs increased during the cultivation, as the amount of uronic acid per wet weight of the samples significantly increased from 0.05 to 0.70 μg/mg per wet weight ($p \leq 0.05$) during the 4-week-cultivation period. The contraction of the gels and decrease of the water content from 95% to 84% at 4 weeks, slightly increased the uronic acid to wet weight ratio. However, the increase in the total amount of uronic acid (from 1.9 ± 1.4 μg/gel at day 1 to 7.0 ± 2.9 μg/gel at 4 weeks; mean ± SD; $p=0.028$) provided evidence for a significantly increased PG production.

The RT-PCR analysis was used to detect changes in the ECM production at mRNA levels. Relative amount of *procollagen α1(II)* and aggrecan mRNA reached its highest level after 1 week of cultivation in the rhCII gels in vitro. At 1 week, the relative quantity of *procollagen α1(II)* mRNA was over 12 times higher, and aggrecan mRNA levels showed over a seven-fold increase when compared with day 1. After the first week, the relative amounts of *procollagen α1(I)* and aggrecan mRNA had declined but remained above the day 1 level.

5.3 ANIMAL MODELS *IN VIVO* (STUDIES III, IV)

To extend the experiments to larger scale living organisms, we performed further tests *in vivo* with chondrocyte-seeded rhCII-hydrogels injected subcutaneously in the backs of nude mice (Figure 8A), and as a repair material for experimentally induced osteochondral lesions in rabbits (Figure 9A).

5.3.1 Macroscopic findings in nude mice

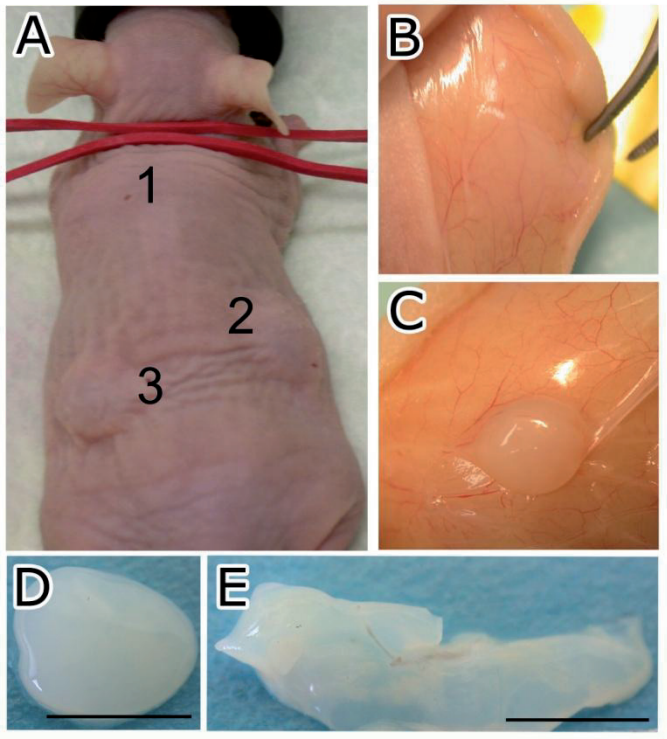

Figure 8. Subcutaneous chondrogenesis in nude mice. Photograph A shows the location of injection sites of (1) rhCII-hydrogel without cells (rhCII-gel); (2) bovine chondrocytes in rhCII-hydrogel (rhCII-cell); and (3) bovine chondrocytes in growth medium (Med-cell). The subcutaneous injections were carried out on anesthetized mice (A). Implantation sites of rhCII-gel (B) and rhCII-cell (C) 6 weeks after the implantation. A closer macroscopic view of the rhCII-cell construct (D) and Med-cell (E) after 6 weeks. Bar = 5 mm.

When the rhCII-constructs were cultivated 6 weeks subcutaneously in nude mouse, the macroscopic appearance of the hydrogels (Figure 7C) clearly changed from being soft, fragile and transparent at the beginning (Figure 7A). The chondrocytes within rhCII-hydrogel implants (rhCII-cell) produced a round or oval piece of whitish, translucent and resilient tissue (Figures 7C, 8C and 8D). The control samples, chondrocytes in medium (Med-cell), also formed a piece of white tissue, but the appearance was thin, uneven and rough (Figure 8E). No macroscopic signs of vascular invasion or abnormal growth of connective tissue were detected around the subcutaneous constructs. Thicknesses of the tissue constructs were statistically different. The tissue produced by the rhCII-cell implants was two times thicker than the Med-cell constructs (p = 0.003). In the control samples, where rhCII-hydrogels were implanted without any cells, the gel structure had mostly dissolved. In this case, only a barely detectable thin layer of material remained at the injection site (Figure 8B). Therefore, these samples were not further analyzed.

5.3.2 Macroscopic findings in the rabbit osteochondral repair model

The macroscopical findings after using the rhCII-hydrogel with autologous chondrocytes in a rabbit cartilage defect model showed that six months postoperatively, the lesion sites were filled with repair tissue, that had a visibly smooth surface for the most part, but the color of the repair was different from the surrounding cartilage area (Figures 7D, 9A). The rhCII-repaired lesions showed almost 100% individual location filling in all animals (Figure 9A), and only one lesion out of 6 showed impaired filling. In contrast, the spontaneous repair was more uneven and contained partially filled areas and impaired filling in half of the animals (Figure 9B) resulting in an average of 79% filling in macroscopical evaluation. Some peripheral cartilage areas of the femoral trochleae were swollen and hyperemic (Figures 9A and 9B). This reaction was apparent in both repair groups and can be considered as a surgical response. The intact control cartilage had a smooth trochlear area (Figure 9C).

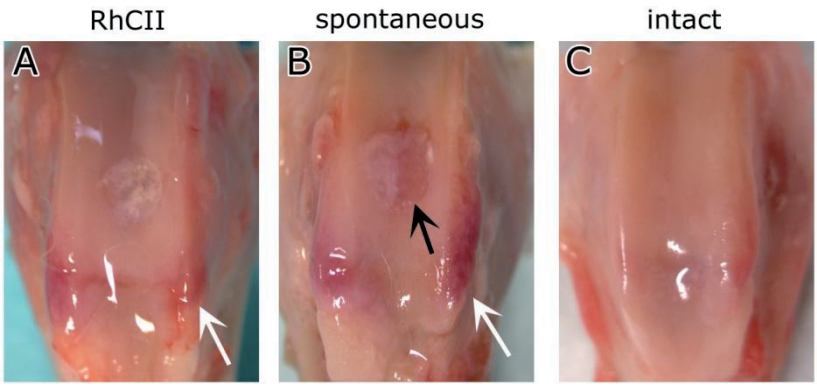

Figure 9. Repair of osteochondral defects in rabbit knee joints. Photographs from A) intact, B) rhCII-gel repair and C) spontaneous repair in the rabbit femoral trochlea after 6 months of operations. The repair site is clearly visible at the rhCII and spontaneous repair sites. In the rhCII-repairs, the defects were filled with repair tissue. In the spontateous repair, the defects were not fully filled with repair tissue, as shown with the black arrow. Some of the joints were hyperemic and swollen(indicated with the white arrows). This reaction was present in both rhCII and spontaneous repair and was not caused by collagen implantation.

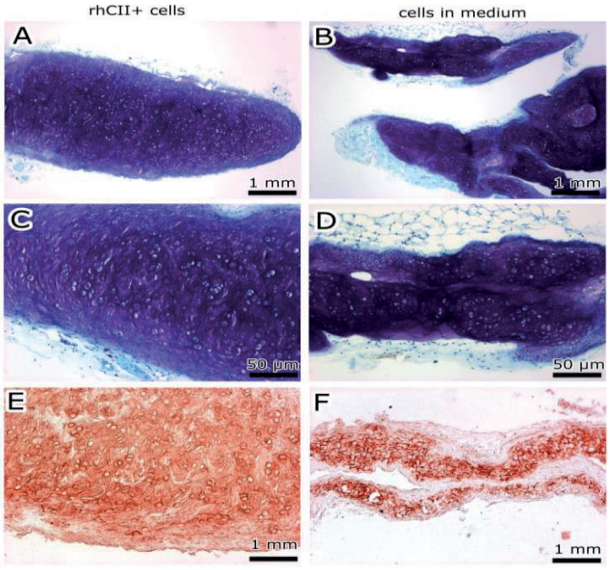

Figure 10. Subcutaneous chondrogenesis in nude mice. Toluidine blue stainings showing extracellular matrix 6 weeks after subcutaneous injections of implants. The chondrocytes seeded in rhCII-hydrogels (rhCII-cell) in different magnifications (A, B). The chondrocytes in growth medium only

(Med-cell) in different magnifications (C, D). Immunostaining for type II collagen showed a strong signal in cell-seeded rhCII constructs (rhCII-cell), particularly around the chondrocytes, suggesting an accumulation of collagen type II (E) in these specimens. Non-cartilaginous areas, negative for type II collagen, were visible in collagen scaffold (hydrogel)-free constructs (Med-cell) (F).

5.3.3 Histological characteristics of repair tissue *in vivo*

Histological analysis of the subcutaneous samples further revealed that the chondrocytes had formed lacunae-like structures (Figures 10 and 11). There were no differences in the numbers of cells contained with the rhCII- and Med-cells constructs. In the rabbit samples, the cellularity in some areas of the repair tissues was higher than in the intact tissue, except that the surface layer was hypocellular in both rhCII assisted and spontaneous repairs (Figures 12B and C). In the tissue adjacent to the repaired area, the cellularity was nearly normal, with mild clustering and slight fibrillation. Some of the repair tissues contained tiny empty spaces, that may have occurred due to localized cell death events (Figure 12C).

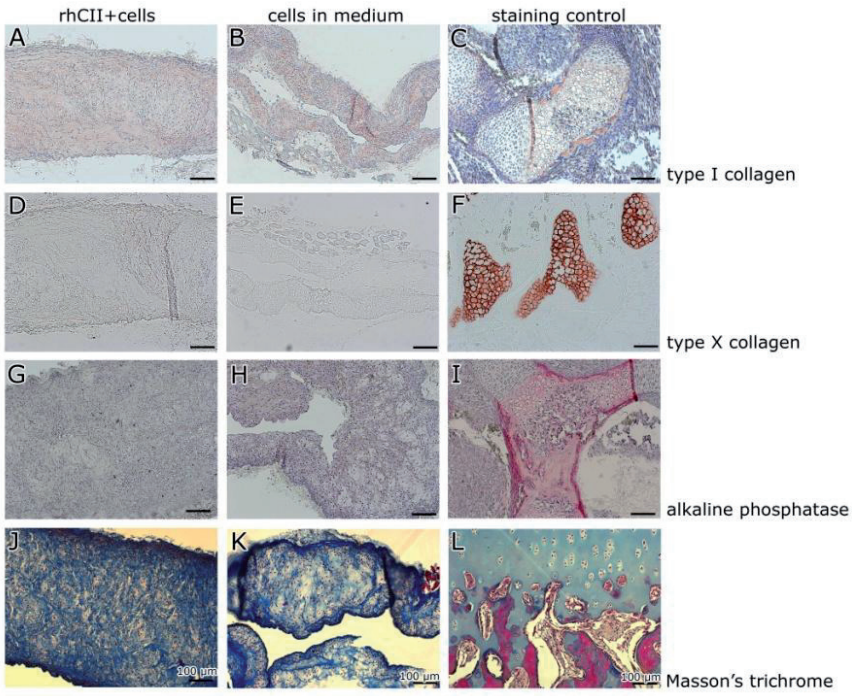

Figure 11. Subcutaneous chondrogenesis in nude mice. Histologic stainings for the subcutaneous samples rhCII-cells, Med-cells and staining controls.

Type I collagen was mildly present in the constructs (A, B). Collagen type X was not detected in the constructs (D, E). Alkaline phospatase (ALP) activity was not detected in the constructs (G, H). Masson's trichrome staining showed no bone specific staining in the constructs (J, K).

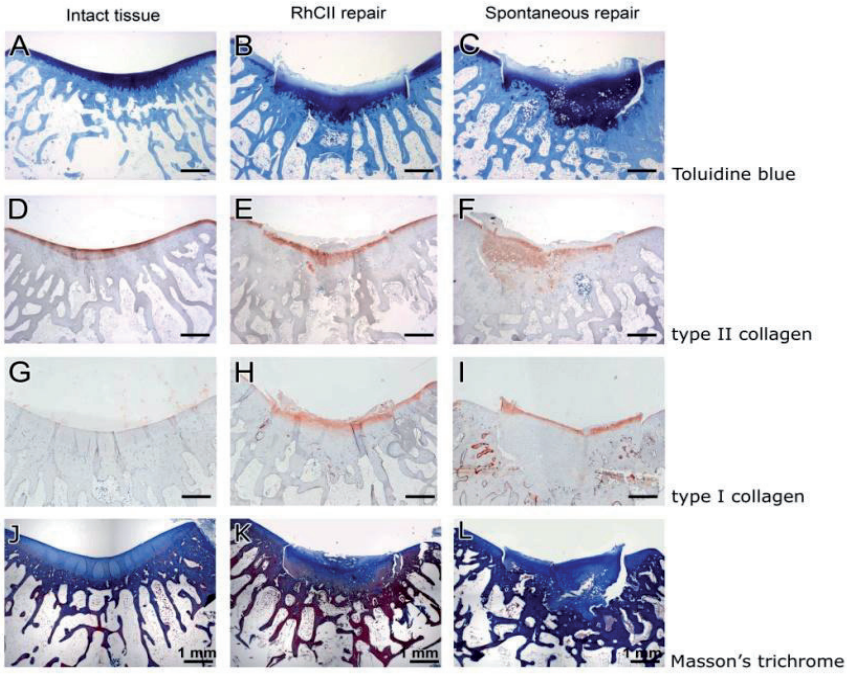

Figure 12. Repair of osteochondral defects in rabbit knee joints. Histological stains of the intact and repaired rabbit joint areas. Toluidine blue (A-C) and type II collagen (D-F) were detected in the intact cartilage and in the deeper zones if the repair tissues. Type I collagen was present in the superficial zones of the repaired tissue (H, I) and in the adjacent cartilage, but not in the intact cartilage (G). Masson's trichrome staining (J-L) indicated the cartilaginous tissue.

5.3.4 ECM production and quality *in vivo*

The chondrogenic character and ECM production of the tissue constructs was examined with toluidine blue staining (Figures 10A and D, and 12D and F). After 6 weeks of subcutaneous cultivation *in vivo*, rhCII-cell constructs produced tissue that was heavily stained with the cationic dye toluidine blue (Figure 10A and C), indicating the presence of abundant extracellular matrix proteoglycans. The findings in subcutaneously cultivated Med-cells were similar, except that the constructs were more

condensed than the rhCII-cell construct (Figure 10B and D). Collagen type II immunostaining was strongly positive in both subcutaneous sample groups (Figure 10E and F), but the cells cultivated in medium were surrounded with type II-negative connective tissue (Figure 10F). Immunohistochemistry revealed that type I collagen was also present in the constructs (Figure 11A and B). To detect whether the chondrocytes had differentiated towards endochondral bone formation, immunoreactivity for type X collagen (Figure 11D and E) and alkaline phosphatase (ALP) was studied (Figure 11G and H). In addition, Masson's trichrome (Figure 11J and K) staining was also carried out. Both stains for hypertrophic or mineralizing cartilage markers showed negative results in subcutaneous rhCII-cell, and Med-cells (Figures 11D-E, and 11G-H). Masson's trichrome staining indicated that the ectopic cartilage implants stained blue, as did normal bovine cartilage, thus indicating no difference from the situation in normal tissue (Figure 11J-K).

A more detailed analysis of ECM production was conducted using the uronic acid analysis and agarose gel electrophoresis for the subcutaneous samples. On the basis of similar uronic acid concentrations, there were no detectable differences in GAG content between the sample groups. Agarose gel electrophoretic analysis (Figure 13A) revealed that in both construct groups, the PGs were primarily large, exhibiting mobility typical for aggrecan molecules, while the amount of small PGs (relative mobility of approximately 0.85-0.90 in proportion to the bromphenol blue front) was low, consistent with previous literature (Lammi 1993, Lammi 1994). The mobility of bovine articular cartilage proteoglycans was similar to rhCII-cell and Med-cell. Chondroitin sulphate C and papain-digested cartilage had faster mobility than the small PG population (Figure 13A). No PG bands were seen from the mouse skin sample extract.

In the rabbit cartilage repair samples, the PGs were also abundantly present and the appearance of the tissue was similar to cartilage in the deeper regions of the repaired region. However, the superficial layer had a low amount of PGs detected with toluidine blue (Figure 12B and C). Immunostaining for type II collagen clearly defined the location of cartilage in the normal, intact, articular cartilage (Figure 12D). In the repaired tissue, it was present in the deeper zone, but not in the hypocellular superficial layer of both repair groups (rh-CII and spontaneous) (Figure 12E and F). The presence of type II collagen in the rhCII repair tissue could be expected, since it was the major material in the implanted gel. However it is still possible that the implanted chondrocytes may have synthesized part of it, as well. However, since

the antibody used for immunostaining cannot distinguish the human and lapine type II collagen, we could not estimate the share of the collagen possibly synthesized by the autologous chondrocytes. In the most superficial layer, the hypocellular part of the repair tissue contained type I collagen (Figure 12H and I) in both repair groups. A thin zone of type I collagen was also present in the tissue adjcent to the repair area, indicating mild tissue deformation also occurred in the surrounding healthy cartilage (Figure 12H and I). In the intact cartilage, type I collagen was not detected, or it existed only in small , diffuse, amounts (Figure 12G).

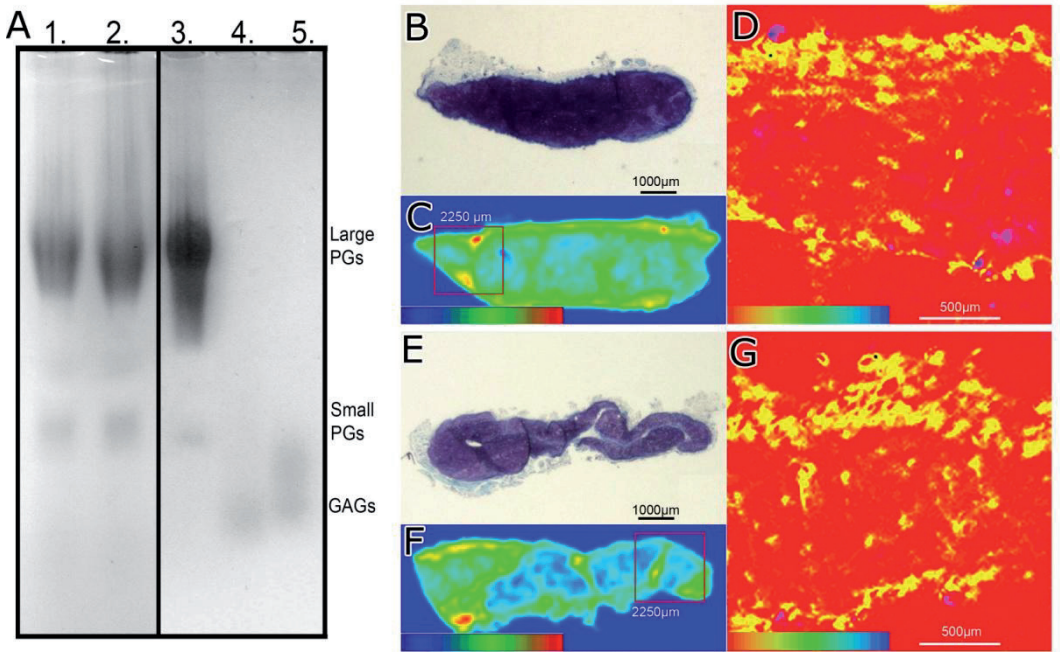

Figure 13. Characterization of subcutaneous chondrogenesis in nude mice. A) Photograph of an agarose gel electrophoresis; Lane 1: rhCII-cell, lane 2: Med-cell, lane 3: bovine cartilage extract, lane 4: chondroitin sulphate C, lane 5: papain-digested cartilage proteoglycans. Toluidine blue-stained sections from rhCII-cell (B) and scaffold-free Med-cell (E) constructs are shown as a reference for FTIR-IS analysis (C and F, respectively). The organization of collagen network, evaluated by parallelism, was evaluated by polarized light microscopy of the rhCII-cell (D) and Med-cell constructs (G). The color scale in C and F indicates the content of collagen (blue is low and red high content), while the scale in D and G indicates the collagen

parallelism (red is low and blue high degree of parallelism). Scale bars B and E = 1000 μm; C and F, 2250 μm; D and G = 500 μm.

The histological samples of the rabbit repair specimens were also viewed and analyzed using histological scoring (modified O'Driscoll method) of the repair tissues (O'Driscoll 1988). The scoring revealed no differences between the spontaneous (score 14.1 ± 2.7, n = 7) and rhCII repaired cartilage (score 12.5 ± 1.4, n = 6). The average score for the intact tissue (n = 7) was 29.1 ± 0.7, while the maximum score was 30. The average cartilage or average repair tissue thickness (± SD) calculated from the histological images was 372 ± 71 μm for the intact tissue, 411 ± 71 μm for the rhCII repair and 273 ± 100 μm for the spontaneous repair sections.

The collagen and PG contents were measured using FT-IRIS. Surprisingly, the use of rhCII did not affect the collagen content, as they were similar in the subcutaneous rhCII-cells and Med-cells samples (Figure 13C and F). In the rhCII repairs the collagen content had higher variation than in the Med-cells. In the osteochondral rabbit repair samples, the total collagen contents were also similar between the rhCII and spontaneous repair, and the values were at similar levels to those in the intact cartilage (Figures 14A, and 15G-I). In comparison to intact tissue, the PG content was significantly lower in both repair groups (Figure 14B). In the rhCII repair group, the amount of PGs was smaller than in the spontaneous repair samples (Figure 14B).

Polarized light microscopy analysis was used to evaluate the arrangement of collagen fibril network of the constructs. In the subcutaneous samples, the degree of parallelism of the collagen fibrils did not differ between the rhCII-cell and Med-cell samples (Figure 13D and G). In the rabbit repair samples, the depth-wise analysis indicated that, in both repair groups, the fibril orientation differed significantly from the intact tissue, (Figures 14D, and 15A-C) through the whole depth of the samples. The overall fibril orientation in the rhCII assisted repair tissue was significantly less perpendicular-to surface than in intact cartilage (Figures 14D, and 15A-C). The parallelism index in the rhCII repair was close to that in the intact cartilage (Figures 14D, and 15D-F), while the spontaneous repair tissue had a more inferior quality in this respect, showing significantly lower values in the middle and deep zones of the repair tissue (Figure 14C).

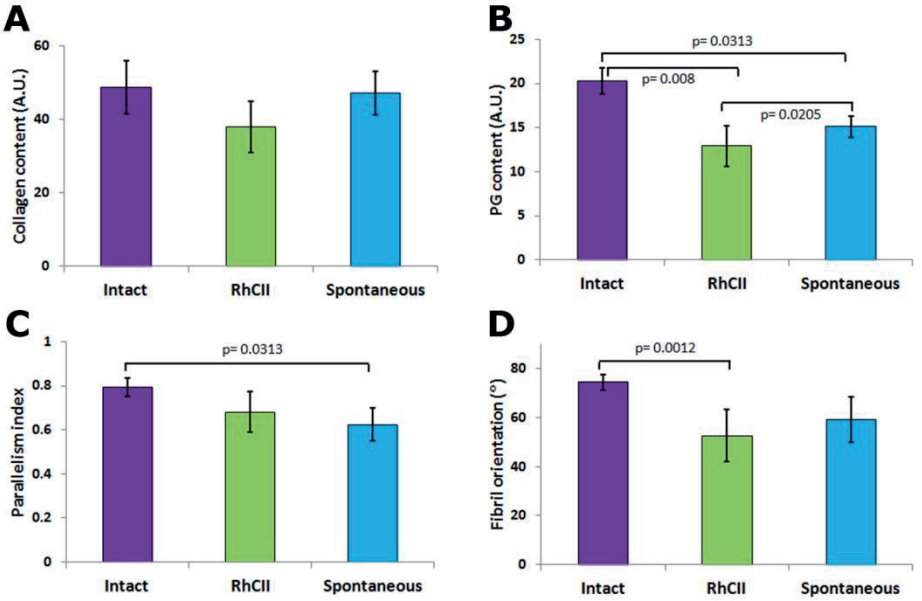

Figure 14. FT-IRIS analysis of the osteochondral repair tissue in rabbit knee joints. The calculated averages of parallelism index (A), the collagen fibril orientation in degrees (B), collagen content (C) and proteoglycan (PG) content (D) in the intact, spontaneous and rhCII-gel repair groups. A.U. = absorbance unit (amide I absorption). Mean ± Confidence interval 95%.

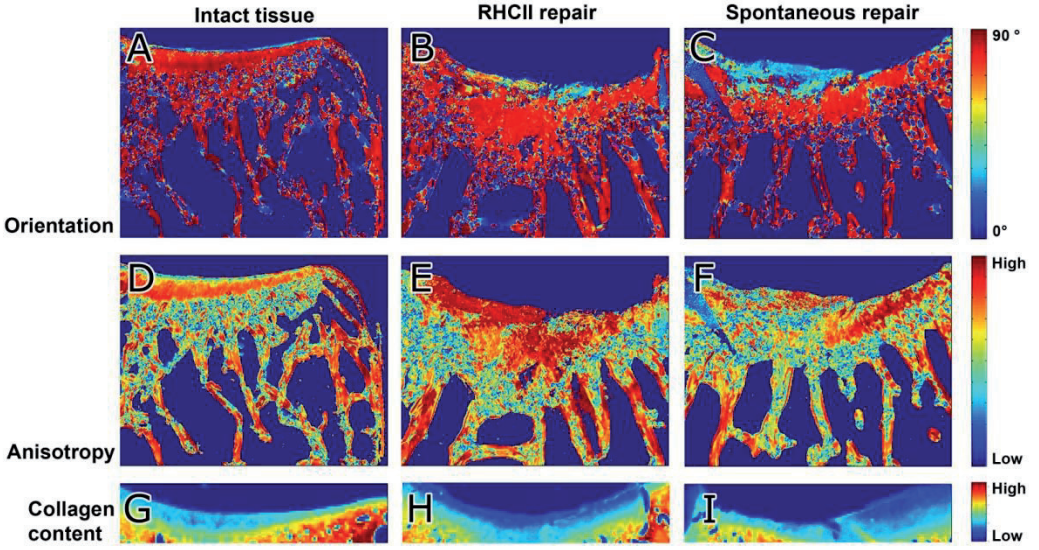

Figure 15. Osteochondral repair in rabbit knee joints. Polarized light microscopy images demonstrating the collagen fibril orientation and parallellism in the intact and repair tissues as indicated above. The red color represents the perpedicular-to-surface and blue color the parallel-to-surface orientation in A-C. In D-F the red color indicates high and the blue color, low

anisotropy. Collagen content distribution in cartilage is shown in G-I in the FTIR images with red and blue colours representing high and low collagen content, respectively.

5.4 BIOMECHANICAL PROPERTIES OF THE RHCII-HYDROGEL CONSTRUCTS

Biomechanical parameters define the functional properties of the articular cartilage. Thus, both equilibrium and dynamic moduli of the tissue samples were analyzed using mechanical testing. The *in vitro* cultivation of the rhCII-hydrogels did not produce tissue hard enough to be measured, so they were not analyzed. Although the macroscopic appearance of the tissue was different between the subcutaneous rhCII-cell and Med-cells constructs, there were no significant differences in the equilibrium or dynamic moduli. The mechanical stiffness did not fully reach that of native bovine articular cartilage (Suh 2000), but was higher than that obtained with *in vitro* cultivation of cellulose sponges.

There were no significant differences in the values of the equilibrium modulus in different groups. This was despite the fact that the spontaneous repair tissue was somewhat softer than both the intact and rhCII assisted repair samples. Similar findings were observed in the dynamic modulus, where the spontaneous repair tissue showed the lowest values (and the differences were insignificant).

5.5 BONE HEALING IN THE OSTEOCHONDRAL REPAIR SAMPLES

It appeared that the bone regeneration was good, 6 months after surgically induced osteochondral injury in rabbits. There were no major differences in the subchondral bone structure between the treatments of repaired cartilage, either by rhCII-assisted or spontaneous repair. The structure of regenerated bone was studied with μCT in two locations: in the vicinity of the injury (VOI1) and deeper in the bone (VOI2). A number of parameters extracted by the analysis software were procured. However, there were no major differences in most of them when compared with intact bone samples. In the VOI1 of the rhCII-assisted repair group, the trabeculae thickness (TbTh) was significantly smaller, and their number (TbN) higher than their respective values, obtained from intact tissue. However, in the VOI2, the trabeculae were thicker but

fewer than in the intact tissue, in contrast to the findings in the VOI1. In the spontaneous repair group, only the trabecular thickness in VOI1 differed significantly from the intact control tissue.

6 Discussion

6.1 FEASIBILITY OF RHCII-BASED SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

6.1.1 Chondrocyte behaviour in rhCII-based scaffolds

The use of type II collagen as a scaffold for chondrocytes in articular cartilage TE has been previously reported (Funayama 2008, Lee 2003, Nehrer 1998). Since, it is the main collagen in native articular cartilage ECM, type II collagen as a scaffolding material would offer a natural environment for the chondrocytes (Buckwalter 1999) that would, via integrin recognition, prevent apoptosis and phenotypic dedifferention (Hirsch 1997). In our studies, we observed that the use of rhCII coating for cellulose sponges improved phenotype preservation of the chondrocytes cultivated in it for 4 weeks. Without rhCII coating, the chondrocytes became elongated *in vitro*, presumably because the cellulose itself did not offer attachment sites for the cells and therefore did not favour chondrocyte growth.

Hydrogels are widely tested for their feasibility as a scaffold for chondrocytes. Because cartilage is a highly hydrated tissue where exchange of nutrients and gases occurs mainly through diffusion, a gel-like environment would have a higher resemblance to the natural composition of articular cartilage. (Drury 2003). Soft rhCII-hydrogels containing viable cells were achieved when they were made combining the rhCII in a HCl containing solution and freshly isolated chondrocytes in growth medium. The cells remained alive during the 4 weeks they were maintained in the cell cultivation conditions *in vitro*, demonstrating that the gel-like scaffold of rhCII allowed sufficient exchange of nutrients, oxygen and waste products. It was also evident that when the gels were made gently by mixing the collagen solution and the cells simultaneously, the chondrocytes were evenly distributed throughout the gel-like scaffold. A smooth cellular distribution is essential when an even textured tissue is desired (Risbud 2002). This has not been achieved often when sponges were used as a scaffold material (Mizuno 2002). The poor ability of the cells to migrate inside rhCII coated collagen sponges was also detected during this work that constitutes this thesis. The poor migration was possibly due to the lack of interconnected pores in the

collagen sponges, which prevents the movement of the chondrocytes deeper into the scaffolds.

Chondrocytes are known to become elongated and fibroblast-like (hallmarks of de-differntiation) when grown as monolayers in culture (von der Mark 1977). This was also shown in our studies, when chondrocytes were cultivated on top of collagen membranes (Tiitu 2008). However, when the cells were embebbed inside 3D material, they tend to maintain their round phenotype and chondrocytic characteristic, chiefly, type II collagen and PG production (Kimura 1984). The phenotype preservation is probably mediated partly via collagen-integrin interactions. It has been reported that mechanical factors also help to maintain chondrocyte phenotype in cartilage TE constructs, as shown in non-collagenous gel-like environments, such as agarose (Kolettas 1995), when combined with or without mechanical loading (Bian 2010).

6.1.2 ECM production in rhCII cultivated chondrocytes

When we cultivated chondrocytes inside the rhCII-hydrogels, we clearly detected cartilaginous formation *in vitro* and *in vivo*, and the chondrocytes produced cartilaginous ECM molecules, type II collagen and PGs, as demonstrated with RT-PCR, histology and biochemical analyses. During *in vitro* cultivation the amount of PGs increased. However, even after 4 weeks, the amount of PGs in the ECM of the tissue-engineered constructs was only 7% of the levels in the normal articular cartilage. The amount of PGs in the osteochondral rabbit repair tissue that received rhCII-coated implants was also lower than in the intact cartilage, but to a lesser extent. The values for PG content were 65% of that obtained from intact articular cartilage, demonstrating that intensive PG production had taken place during the 6-month-recovery period. The amount of collagen in the rhCII assisted osteochondral repairs in the rabbits was also lower compared to intact cartilage, but at an insignificant level. Since it is difficult to differentiate between collagens of different species reliably, and because we did not use any predefined markers in the rhCII collagen, we can confirm neither how much of the type II collagen was produced by the implanted chondrocytes, nor how much of it was of rhCII origin. However, RT-PCR performed on the *in vitro* samples revealed increased mRNA levels for type II procollagen, and histology for type II collagen showed increased pericellular staining, indicating active type II collagen synthesis. Therefore, it is reasonable to assume that some of the type II collagen detected in the rhCII-repaired tissue was produced by the implanted lapine chondrocytes, although part of it could originate from the implanted rhCII itself.

6.1.3 Water content and contraction in rhCII-hydrogels

In normal articular cartilage, the water content of the tissue is estimated to be 65-80% (Newman 1998). In the rhCII-hydrogels, combined with chondrocytes, the water content was first 95%, but during the *in vitro* cultivation for 4 weeks, this concentration decreased to 84%, consequently approaching the water composition in the normal articular cartilage. When the rhCII-hydrogels combined with chondrocytes were cultivated for 6 weeks subcutaneously in nude mice, the water content of the tissue constructs was 82%. This result implies that the water loss is greatest during the first 4 weeks and the water content in the *in vivo* cultivated samples remains at the same levels as that found in intact cartilage. Since, in the native articular cartilage, PGs are responsible for water retention (Mow 1999), their elevated amounts, produced by the implanted chondrocytes, presumably affects water content in tissue-engineered constructs, helping it reach levels similar to those described for normal articular cartilage.

Decreasing water content during cultivation also leads to the contraction of the gels. The diminished size of collagen gels over time, has been reported previously (Galois 2006). Experiments with fibroblasts have shown that this process is affected by many factors. This includes, but is not limited, to collagen concentration, cell density, and growth factors (such as transforming growth factor-β and platelet derived growth factors) (Chen 2006, Malemud 1994). In both our *in vitro* and *in vivo* studies, we noticed that when rhCII-hydrogels were cultivated without cells, they dissolved during cultivation. However, in a previous study by Hunter and coworkers, it was noticed that when the type I collagen gels were cultivated without cells, the gels did not dissolve nor contract, in contrast to the contraction of 37% when bovine chondrocytes were present in the gels (Hunter 2002), indicating that the contraction is somehow related to cellular interactions. The reasons and consequences of the contraction are not fully understood, but obviously when even filling of the defect is desired, a large-scale contraction is an unwanted result. Contraction in a cartilage repair could be avoided using precultivated constructs where the major size reduction has already occurred (Kawamura 1998). Alternatively, specific treatments that inhibit the contraction, such as dihydrocytochalasin B (Galois 2006), or photochemical cross-linking could be used (Ibusuki 2007). However this could have the effect of inhibiting the elimination of excessive tissue. Excess tissue may interfere with proper functioning of the cartilage and joint.

6.1.4 Maintenance of the hyaline cartilage phenotype

All the TE methods that use isolated chondrocytes or differentiated MSCs rely on the finding that the detached cells are capable of proliferation, growth, and ECM production (Brittberg 1996), although when existing inside the ECM in their innate surrounding, the mature chondrocytes rarely express these functions (Buckwalter 1998b). Consequently, it can be speculated that the proliferation and growth detected in the isolated and cultivated chondrocytes are features of a process that resembles the earlier developmental stages of cartilage. Accordingly, there is a possibility that when growth and proliferation is initiated in mature chondrocytes, this process can lead to chondrocyte hypertrophy and ossification (Dell'Accio 2001), those actions that take place during the normal development in transient cartilage (Goldring 2006). In our studies using the nude mice and subcutaneous cultivation of rhCII and chondrocytes, we tested our specimens using different markers for hypertrophic chondrocytes and bone. We did not notice signs of hypertrophy or ossification when using the alkaline phosphatase activity, type X collagen expression and Masson's trichrome staining as markers in our subcutaneous samples of rhCII and chondrocytes.

In addition, no signs of vascular formation, inside the subcutaneous constructs, were detected. Nevertheless, it was evident that the implanted chondrocytes inside the rhCII gels were able to acquire sufficient nutrients and oxygen from the surrounding subcutaneous fibrous tissue because of the constructs showed growth and maturation. In native articular cartilage chondrocytes obtain the essential supplements from the synovial fluid via diffusion (O'Hara 1990), and in the subcutaneous environment, the extracellular fluid must have served this purpose.

6.2 CLINICAL IMPLICATIONS

6.2.1 RhCII-hydrogels improved filling in cartilage lesions

If the transplanted chondrocytes alone are capable of tissue formation after ACT procedure (Brittberg 1994), it is highly probable that the repair procedure would benefit from embedding the cells inside a biomaterial, where the filling of the lesion site and chondrocyte distribution would be enhanced (Figure 16). This is the main hypothesis behind all TE techniques using biomaterials. In the course of the studies of this thesis, it was noticed that the subcutaneously cultivated constructs in nude mice were more even when rhCII was used as a scaffold for chondrocytes in comparison to cells without any scaffolding material. Accordingly, in the

osteochondral repair study in the rabbits, the rhCII-hydrogel assisted repaired lesions showed more even filling than the spontaneous repair when they were macroscopically evaluated, 6 months post-operatively. In the rhCII assisted repaired tissue, only 1 lesion of 6 showed impaired filling. The average filling in the repaired osteochondral defects in rabbits that received rhCII-hydrogel implants with autologous chondrocytes was 99%. In comparison, spontaneous repair samples showed impaired filling in half of the animals, resulting an average of 79% filling in macroscopical evaluation. Comparable results (88 ± 6% filling) were obtained in a study where autologous chondrocytes and type II collagen scaffold (sponge) were used to repair canine chondral defects (Lee 2003).

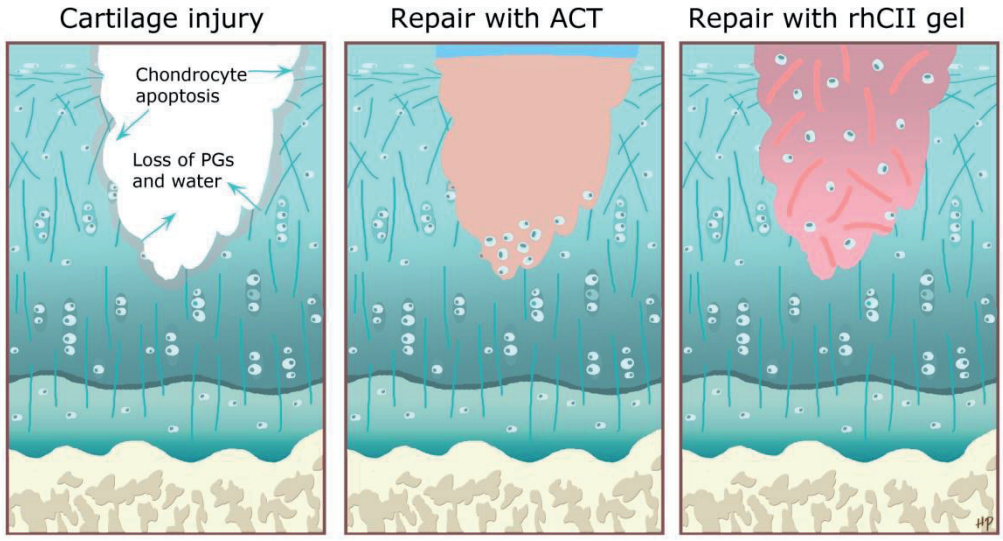

Figure 16. Schematic presentation of cartilage injury (chondral), and repair of cartilage injury using autolologous chondrocyte transplantation (ACT), and rhCII gel combined with chondrocytes.

6.2.2 Quality of the rhCII repair tissue and fibrillation

Although the filling was better in the repaired osteochondral lesions in the rabbits, which received rhCII-hydrogel implants, the quality of the new tissue was not totally satisfactory. Whilst, the deeper regions of the rhCII repaired cartilage contained type II collagen and PGs, the superficial layer lacked these components and resembled fibrous tissue in that it was observed to contain type I collagen immunoreactivity and

hypocellular regions. Fibrillation of the superficial layer is a persistent problem in cartilage repair (Minas 2011). The reasons behind this phenomenon are still largely under speculation and, therefore, when fibrillation occurs, it remains incurable at the moment. Superficial fibrillation might be a consequence of the gradual regression of immature repair tissue and chondrocytes (that had initially attempted to produce hyalinous ECM). On the other hand, it might have been due to an immediate loss of or a weakening in the chondrocytes ability to produce ECM. In this case, the chondrocytes are unable to produce normal hyaline ECM after injury, and instead fibrous tissue is generated.

The injured cartilage becomes more vulnerable to degeneration and development of posttraumatic osteoarthritis (Brown 2006). The degradative enzymes (Kurz 2005, Tchetverikov 2005) and cytokines, such as interleukin 1 and TNF-α (Goldring 2000) secreted by chondrocytes after injury, affect the whole joint. It has been shown that immature tissue in younger animals, is even more vulnerable to matrix destruction after cartilage injury (Kurz 2004, Levin 2005). In the case of cartilage regeneration after injury, the newly synthetized repair tissue is also immature, and more sensitive to mechanical loading. It is possible that the collagen structure of the immature repair tissue becomes more easily damaged, leading directly to loss of PGs or a progressive inability to hold them inside the tissue. The loss of PGs is also affected by several degradative enzymes, produced by the chondrocytes after injury (Kurz 2005). In our osteochondral rhCII assisted repair samples, the collagen content was lower only in the superficial layer (overall collagen contents were not statistically different from intact specimens), but the amounts of PGs were significantly lower throughout the repair tissue thickness. We did not use any enhancing substances in our studies to prevent the loss of neither PGs nor GAGs, or stimulate their production. The use of enzyme inhibitors such as the MMP inhibitor, CGS 27023A (DiMicco 2004), caspase inhibitors (D'Lima 2001b) or antioxidative substances (Del Carlo 2002) to inhibit chondrocyte apoptosis, have been shown to decreaseGAG loss or chondrocyte cell death after injury.

After injurious compression *in vitro*, the expression of type I collagen has been detected to increase by 2.5-fold in 24 hours (Lee 2005). In our rabbit osteochondral repairs, a layer of type I collagen was detected in the most superficial layer of the rhCII-assistedand spontaneously repaired tissue. It may be that after articular cartilage damage, the repair program in the remaining chondrocytes activates and results in an attempt to build up fibrous “scar-like” repair tissue into the lesion site and this ultimately leads to the infamous poor quality of the spontaneous repair tissue. Nevertheless, the use of adult (dissected from cartilage biopsy and

enzymically isolated) chondrocytes as a cell source for cartilage TE is propably not the best option, allthought they are currently widely used. In addition to the harsh digestion protocols, monolayer expansion of the adult chondrocytes causes dedifferentiation that will generate ageing in the chondrocytes and compromise the normal phenotype and function (Khan 2008). The fact that we used monolayer cultivated adult chondrocytes in our rabbit experiment might have affected the slightly fibrillated outcome in the rhCII repaired cartilage tissue. However, because the spontaneous repair tissue was equally fibrillated, the initial condition of the transplanted cells is not the only problem. In spontaneously repaired tissue, the cells responsible for the growth of new tissue (MSCs, progenitor cells, migrating chondrocytes) are affected by the aforementioned cytokines as well as other mediators that are present in the injured joint. Similar conditions affect the transplanted chondrocytes, if no measures to prevent the actions of these extrinsic agents are taken.

In our osteochondral repair study we noticed that a thin layer of type I collagen was covering also the adjacent tissue of the repaired cartilage (both in spontaneous and rhCII assisted repair tissues). In the previous literature the occurrence of type I collagen after articular cartilage injury has been rarely reported, even in the control specimes of spontaneous repairs. The production of type I collagen is strongly associated with OA (Gebhard 2003, Martin 2001). Its expression has been shown to be elevated after mechanical injury *in vitro* as well (Lee 2005). The finding of type I collagen in the adjacent tissue surrounding a cartilage lesion therefore implies that the injury is causing extended changes also to the surrounding tissue and not only in the lesion area. At the macroscopic level, the joints of the rabbits appeared to be partly swollen and hyperemic at the time of sample preparation, 6 months post-operatively. This phenomenon took place in both the spontaneous and rhCII-induced repair groups. This implies that the rhCII material did not cause collagen-specific inflammation. At this moment, it is unclear, whether the swelling and incidence of type I collagen indicate the early development of post-traumatic OA, or do they represent normal reversible changes in the joints after trauma.

The problems relating to transplantation of adult chondrocytes in cartilage repair procedures might be overcome in the future with the use of MSCs or chondrogenic progenitor cells, with combination of suitable growth factors (Gerter 2012). In addition, the use of specific anti-inflammatory agents, in combination with cell transplantation, could hinder the fibrillation and degradation of the repaired cartilage. Additionally, the use of artificial surface layer, such as collagen

membrane, to cover the repaired area, could diminish superficial fibrillation. No periosteal flap on top of the gel repair constructs was used in the rabbit study. This is because of the reported tendency to overgrowth when periosteum has been used (Recht 2003) and the difficulties in operating such small scale surgery in rabbit joints.

6.2.3 Collagen degradation and immunogenicity

The degradation of rhCII containing material, which was not separately studied in the thesis, has also an effect to the composition of the repair structure. Studies related to collagen fillers in soft tissue augmentation showed that the life time for these fillers, under patients's skin, was from 3 to 18 months (Baumann 2006). In our nude mouse study, where plain rhCII-hydrogel was injected subcutaneously, a thin layer of material was observed at the injection site after 6 weeks, and most of the initial gel volume was dissolved at that time. The increase in the MMP 13 expression of chondrocytes cultivated in the collagen (type I and III) containing gels shows that the chondrocytes initiate collagen degradation when embedded inside gels (Galois 2006). Generally it is assumed that the initially implanted collagen biomaterial is degraded in the course of time (Drury 2003), although this has not been demonstrated taking place for chondrocytes or cartilage tissue.

The supposedly higher immunogenicity of type II collagen has raised concerns, since it may induce arthritis when injected into mice or rats (Brand 2007), and antibodies to type II collagen are present in the rheumatoid arthritis in human patients (Cremer 1998). However, the relationship between the type II collagen and autoimmunity response is not straightforward as the induction of the disease in animal models requires use of an adjuvant that promotes the inflammatory reaction (Brand 2007) and the connection between the cause and result is unclear. Secondly, there is no evidence that induced autoimmunity in humans could result from type II collagen implantation, as no such data exists (Lynn 2004). Finally, type II collagen gel in rabbits (Funayama 2008) and type II collagen scaffolds in dogs (Lee 2003, Nehrer 1998) do not cause detectable immunological reactions, as well.

6.2.4 Integration of repair tissue

The integration of the rhCII assisted osteochondral repair tissue in the rabbits into the subchondral bone was judged to be good. However, lateral integration into the adjacent cartilage was incomplete. Earlier data have demonstrated that the integration of the repair tissue with the adjacent healthy cartilage is problematic (Khan 2008, Shapiro 1993).

Several factors are known to inhibit or weaken the integration of a repair transplant: excessive degradative processes taking place in cartilage and surrounding tissues, and the anti-adhesive properties of PGs and other molecules, such as lubricin, in the synovial fluid (Englert 2005, Schaefer 2004). The border area surrounding the defect almost inevitably contains dead cells, surrounded by extensive ECM, which could, at least partially, be removed by degrading enzymes, such as chondroitinase (Hunziker 1998) to enhance chondrocyte mobility and improve the integration of the immature constructs into the host cartilage. In summary, it is possible that some part of the disintegration detected in the rabbit study in the osteochondral repair samples was an artefact due to the tissue processing protocol for histology.

6.2.5 Collagen fibril organization and mechanical properties

Collagen fibril organization is subjected to alterations during growth and maturation (Julkunen 2010). In mature, intact cartilage, collagen fibrils assembly and form arch-like structures. Of course, the collagen fibrils are randomly organized in the freshly manufactured gels, such as rhCII-hydrogels with chondrocytes. The knowledge about how collagen fibrils organize and form large orientated structures during the normal development and maturation or during the repair process is largely unkown, currently. For that reason, there are no specific treatments to improve the organization of the collagen fibrils, but instead it is only anticipated that the correct arrangement would be achieved after transplantation of TE construct or biomaterial. The overall fibril orientation in the rhCII assisted osteochondral repair tissue, post-operatively in the rabbits, was significantly less perpendicular, with respect to the surface, than in the intact cartilage. This shows that the collagen fibrils were still more randomly distributed than in intact cartilage after 6 months. In the spontaneous repair model, the average fibril orientation was not statistically different from the intact tissue, indicating that newly synthesized collagen fibrils were in an orientation that closely resembled normal tissue, when no scaffolding matrix was used. When the parallelism index, which evaluates whether the fibrils run in the same direction or not, was evaluated, the rhCII showed more similarity with the intact tissue than the spontaneous repair. In the spontaneously repaired tissue samples, the parallelism index was significantly lower than in the intact cartilage, especially in the middle and deep zones. The random organization of collagen fibrils resembles the earlier development stages of cartilage, rather than the mature tissue where distinct zonal organization is predominant (Julkunen 2010). It is an

open question whether the maturation process of collagen fibrils can take place distinctively inside the repair tissue in the adult cartilage leaving the intact tissue untouched in the cases of injured cartilage. It is also not clear how the process of degradation of the implanted scaffolding material alongside ECM production and modification would function in reality. Although the collagen fibril organization and properties are an important factor of intact cartilage, they are seldom evaluated in the repair studies, and no such data exists from human patients, to our knowledge.

Even though the structural characteristics of collagen fibrils and ECM differed from the intact cartilage in the repairs of rabbit osteochondral injuries, the mechanical properties of the rhCII assisted repaired cartilage were not statistically different from the intact cartilage in terms of equilibrium and dynamic moduli. The rhCII assisted repaired cartilage had slightly improved mechanical properties than the spontaneously repaired cartilage (the difference was insignificant). Usually there is correlation between the quality and mechanical properties of the cartilage (Julkunen 2009). However in our results, the inferior quality of the rhCII assisted repaired cartilage tissue (especially in the superficial layer), as measured in histology, did not result in any significant worsening of the mechanical function of the repaired tissue. This might be partly explained by the different orientation of collagen fibrils, in the repaired cartilage that received rhCII-hydrogel implants that might cause stiffening of the tissue. Another explanation might be the challenges faced during the measuring protocol. The mechanical testing we used to analyze dynamic and elastic properties of the samples requires positioning of preset load onto the cartilage, prior to the indentation protocol. The surface irregularities and softness of the repair tissue in the injured cartilage complicated the accomplishment of this task and resulted in a flattening of the superficial tissue before the measurements took place. Due to this, the mechanical results of the repair tissue possibly represent the mechanical properties of the deeper areas of repair tissue that contained also histologically good-quality tissue, and excluding the fibrillated superficial layer.

6.2.6 Translational results and the source of collagen

It is known that an animal model with repair characteristic similar to the human species does not exist (Minas 2011), and the animal experiments that are used by others and in this thesis project, offer only translational results. In subcutaneous rhCII assisted cultivation in the mice the constructs showed smooth surfaces, a result which might predict also

improved surface properties in cartilage repair. However, despite the initially promising results, the surfaces of osteochondral defects in the rabbit joints became microscopically fibrillated and the repair outcome was not much improved compared to the results of spontaneous repair when the rhCII was implanted. This could be partly explained to be due to species specific differences. It ha sbeen shown that rabbit cartilage differs from human in terms of thickness, resident cell population density and repair capacity (Minas 2011). For example, in rabbits spontaneous cartilage repair is generally good when compared to that observed in human patients. Therefore, it is difficult to estimate the exact beneficial effect the rhCII-hydrogel could provide in humans for cartilage regeneration as a scaffold biomaterial. It is also possible that the full benefit of using human type II collagen is not utilized in the animal models due to inter species differences.

If collagen is used as a scaffold for chondrocytes in articular cartilage reconstruction for patients, hypothetically the best option would be to use collagen of a human type. The collagens that are currently used for cartilage TE are of animal origin, and therefore raise questions about safety (Lynn 2004), for example the use of bovine collagens poses a possible risk to exposure to cattle derived diseases such as bovine spongiform encephalopathy (Matarasso 2006). The risk of contagious disease is evident also with human derived biomaterials. The type I and III collagens have been extracted from cadavers or from fibroblast cultivations and used as skin fillers. It is not known if these were properly screened for human pathogens capable of horizontal transfer. The material itself may be hazardous, due to adverse reactions in heterologous humans. These human derived collagens have been reported to cause only mild adverse reactions, such as bruising. (Baumann 2006). Besides cadaver-originated collagens, it is otherwise difficult to obtain human type II collagens, except using recombinant collagens. Therefore, rhII collagen could offer a safe and reliable source as a filler material to be used in clinical operations, such as articular cartilage TE, instead of the currently used animal collagens.

7 Summary and conclusions

The results in the present study evaluated the feasibility of rhCII as a scaffold for articular cartilage repair. Various experimental methods were utilized to assess suitable formulations for the delivery of rhCII to cartilage damage. Its suitability was measured in terms of cellular, ECM and biomechanical properties. The main findings and conclusions can be summarized as follows:

1. When chondrocytes were grown in rhCII-based material, they were viable and able to produce articular cartilage specific ECM: mainly type II collagen and PGs.
2. The optimal formula for rhCII was a gel-like scaffold, which allowed for an even cell distribution and growth as well as ECM production. The gel-like scaffolding material of type II collagen could be further improved by combining with other ECM molecules or growth factors in the future studies.
3. The use of rhCII as a gel-like scaffold for chondrocytes in osteochondral repairs improved the filling of the lesions and resulted in slightly improved mechanical properties in comparison to spontaneous repair. However, the use of rhCII in the osteochondral repair procedure did not solve the problems of incomplete integration of the repair tissue into adjacent cartilage and fibrillation in the superficial repair cartilage.
4. Therefore, before the rhCII can be used on human patients, further studies concerning enhancing the integration and surface properties of the repair tissue are needed.

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The Use of Recombinant Human Type II Collagen for Articular Cartilage Tissue Engineering

Articular cartilage injuries require surgical intervention in order to regenerate repair tissue. In this thesis, tissue engineering of cartilage was studied using a novel biomaterial of recombinant human type II collagen (rhCII) as a scaffold for chondrocytes. This work confirmed the feasibility of rhCII to be used for cartilage tissue engineering. When osteochondral defects were repaired with rhCII, slight improvement was detected in the filling of the lesions and in mechanical properties of the repair tissue in comparison to spontaneous healing. These results also emphasize the complex nature of cartilage repair and the importance for further studies.

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